

ANNALES MEDICINAE EXPERIMENTALIS ET BIOLOGIAE FENNIAE

REDACTORES:

E. MUSTAKALLIO
(TURKU)

U. UOTILA ARMAS VARTIAINEN
(HELSINKI) (HELSINKI)

ALVAR WILSKA
(HELSINKI)

A. I. VIRTANEN
(HELSINKI)

EDITOR

K. O. RENKONEN

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY
OF HELSINKI

SENSITIVITIES OF BETA-HAEMOLYTIC STREPTOCOCCI

FOLLOW-UP STUDIES WITH SCARLET FEVER PATIENTS TREATED
WITH DBED-PENICILLIN OR TETRACYCLINE

by

I. P. PALVA and W. J. KAIPAINEN

(Received for publication September 2, 1958)

Reports on the treatment of scarlet fever (Forssell, Klemola) with DBED-penicillin have been published previously (1955) from the Aurora Hospital. A single dose of penicillin combination containing DBED-penicillin was able to produce a good clinical and bacteriological improvement in the initial phase, but streptococci were established at follow-ups in up to 11 per cent of the cases. A concurrent slight increase in the AST titre was observed. In the tetracycline-treated series of the same hospital (Klemola 1957) the initial clinical and bacteriological result was good in 95 per cent of the cases, but in c. 50 per cent streptococci reappeared 3 weeks after the initiation of the therapy and the mean AST titre rose simultaneously higher than in the group treated with DBED-penicillin. Hence it may be assumed that both DBED-penicillin and tetracycline are capable of clearing streptococci from the pharynx for a short time but that tetracycline in particular does not achieve in the tonsils an antibiotic effect capable of destroying the streptococci in the deeper tissue layers.

We are indebted to the former director of the Municipal Bacteriological Laboratory of Helsinki, Prof. Nils Oker-Blom, M.D., for placing the cases at our disposal.

The series referred to consisted of a total of 200 cases of scarlet fever all of which showed on admission typical rash and beta-haemolytic streptococci in the throat or nose. Approximately 25 mg/kg of tetracycline (Achromycin Lederle) was administered daily to 61 patients and approximately 12 mg/kg body weight to 39 patients; all the patients received a fiveday course of the therapy. 100 patients were given on the day of admission an intramuscular injection of a penicillin combination containing 300 000 units of DBED-penicillin, 150 000 units of procaine penicillin G and 150 000 units of potassium penicillin G (Penadur 6-3-3 Leiras). A DBED-penicillin injection of 1 200 000 units was administered on the sixth day of hospitalisation. The dose was repeated two weeks later at the first follow-up.

The present study was an examination of the sensitivities of the streptococci in the relapsing cases of the above material to penicillin and tetracycline, with special attention to a possible increase in resistance. The authors also want to obtain indications as to the suitability of the mean blood concentration values employed in resistance determinations as comparative data for the assessment of the antibiotic effect *in vivo* in, here, tonsillitis.

The present writers' series comprised streptococcal strains which were isolated from the 6 DBED-penicillin-treated and the 32 tetracycline-treated patients in whom streptococci of the above-mentioned groups re-appeared.

The sensitivities of the bacteria were determined against potassium penicillin G and tetracycline by using the two-fold plate dilution method with blood agar, pH 7, as the culture medium. The bacterial strains were stored in dry ampules, and 2 bloodbroth passages were cultivated from them prior to the sensitivity determination.

RESULTS

Table 1 shows that at the inception of therapy all the streptococci strains could be taken as very sensitive to penicillin: 1 strain was sensitive to 0.05 UI/ml of penicillin and the other 6 strains were sensitive to 0.02 UI/ml or less. After the termination of therapy 6 days later streptococci were present in 3 cases. In 2 of these cases sensitivity determinations showed that the bacterial strains were still highly sensitive to penicillin. A new sample was

TABLE 1
 FOLLOW-UP EXAMINATIONS OF THE PENICILLIN SENSITIVITIES OF β -HAEMOLYTIC STREPTOCOCCI IN SCARLET FEVER PATIENTS TREATED INTRAMUSCULARLY WITH A PENICILLIN COMBINATION (6-3-3) CONTAINING 300 000 UNITS DBED-PENICILLIN, 150 000 UNITS PROCAINE PENICILLIN G AND 150 000 UNITS POTASSIUM PENICILLIN (*L-A*) ON ADMISSION AND 600 000 UNITS DBED-PENICILLIN (*L-A*) ON DISCHARGE

Patient	On Admission			On Discharge 6+H Day			3-Week Follow-up			7-Week Follow-up			
	Sex	Age	Type	Sensi-tivity IU/ml	Treatment	Type	Sensi-tivity IU/ml	Treatment	Type	Sensi-tivity	Treatment	Growth of Strep-tococci	
B. K.	♂	3	A	22	≥ 0.02	*6-3-3*	600 000 IU	A 22	≥ 0.02	*L-A*	1 200 000 IU	A 22	≥ 0.02
A. K.	♂	3	A	12	≥ 0.02	*6-3-3*	600 000 IU	A 12	≥ 0.02	*L-A*	1 200 000 IU	A 12	≥ 0.02
M. L.	♀	5	A	22	0.05	*6-3-3*	600 000 IU	—	—	*L-A*	1 200 000 IU	A 22	0.05
J. V.	♂	5	A	6	≥ 0.02	*6-3-3*	600 000 IU	—	—	*L-A*	1 200 000 IU	A 6	≥ 0.02
H. N.	♂	6	A	22	≥ 0.02	*6-3-3*	600 000 IU	—	—	*L-A*	1 200 000 IU	A 22	≥ 0.02
A. K.	♀	7	A	12	≥ 0.02	*6-3-3*	600 000 IU	+	+	*L-A*	1 200 000 IU	A 12	≥ 0.02

TABLE 2

FOLLOW-UP EXAMINATIONS OF THE TETRACYCLINE SENSITIVITIES OF β -HAEMOLYTIC STREPTOCOCCI IN SCARLET FEVER PATIENTS TREATED PERORALLY FOR 5 DAYS WITH TETRACYCLINE

Patient		On Dismission		Daily Dose mg	On Discharge	3-Weeks Follow-up	
Sex	Age	Type	Sensitivity $\mu\text{g/ml}$			Type	Sensitivity $\mu\text{g/ml}$
R. A.	♀ 1	A 27	1.5	250	—	A 12/27	1.5
J. I.	♂ 3	A 22	1.5	350	—	A 22	1.5
S. A.	♂ 3	A 22	0.8	200	—	A 22	0.8
J. K.	♂ 3	A 12/27	1.5	200	—	A 12/27*	1.5
M. K.	♀ 3	A 12/27	1.5	200	—	A 12/27	1.5
E. K.	♀ 3	A 22	1.5	350	—	A 22	—
C. B.	♀ 4	A 22	3	200	—	+	—
S. M.	♀ 4	A 12/27	3	200	—	A 12/27*	6
T. H.	♀ 4	A 1	6	200	—	A 1	3
T. V.	♀ 4	A 1	6	200	—	A 1	6
P. K.	♀ 4	A 27	3	300	—	A 25	3
T. S.	♂ 5	A 22	1.5	200	+	A 22	3
H. A.	♂ 5	A 3	1.5	200	—	A 25	3
R. P.	♀ 5	A 22*	1.5	350	—	A 22*	1.5
R. T.	♀ 5	A 22	1.5	350	—	A 22	1.5
R. H.	♀ 6	A 1	3	300	—	A 1	3
L. I.	♀ 6	+	1.5	500	—	+	1.5
E. K.	♀ 7	A 3	3	500	—	A 3	3
R. K.	♀ 7	A 22	—	500	—	A 22	1.5
B. H.	♂ 7	A 3	1.5	500	+	A 3	1.5
L. E.	♂ 8	A 3	3	300	—	+	1.5
I. V.	♀ 8	A 3	3	500	—	A 3	3
K. R.	♀ 8	A 12	3	500	—	A 12	3
T. H.	♀ 9	A 12	3	300	—	A 12/27	1.5
A. B.	♀ 9	A 12	0.8	400	—	A 12	1.5
T. F.	♀ 9	A 22	1.5	500	—	A 22	1.5
R. L.	♀ 9	A 12	3	500	—	A 12	1.5
I. L.	♀ 10	A 3	3	500	—	A 3	3
L. T.	♀ 11	A 3	3	600	—	A 47	3
R. B.	♂ 12	A 12	3	400	A 12	A 12	3
K. K.	♂ 13	A 3	3	400	—	A 3	3
J. N.	♂ 19	A 3	3	1 000	—	A 3*	—

* Strain isolated from nose.

TABLE 3
THE SENSITIVITIES TO PENICILLIN AND TETRACYCLINE OF 153 BETA-HAEMOLYTIC STREPTOCOCCI STRAINS ISOLATED FROM SCARLET FEVER PATIENTS

IU/ml	Group Treated with Penicillin Sensitivity to			Group Treated with Tetracycline Sensitivity to		
	Penicillin			Tetracycline		
	Number of Strains	Per cent	$\mu\text{g}/\text{ml}$	Number of Strains	Per cent	IU/ml
0.02	32	89	0.4	1	3	0.02
0.05	4	11	0.8	3	8	0.05
0.1	—	—	1.5	22	61	0.1
0.2	—	—	—	10	28	0.2
	36	100		36	100	
					117	100
						117
						100

taken c. 3 weeks after the inception of therapy and streptococci types corresponding to the original strains were found in all the 6 cases. These streptococci were still sensitive to penicillin. At the 7-week follow-up streptococci were still found in 3 cases.

Table 2 analyses 32 cases treated with tetracycline. The original test revealed the sensitivities to tetracycline to be roughly the same; after a 5-day course of 0.8—6 µg/ml, streptococci were still present in 3 cases. At the follow-up 3 weeks later streptococci were demonstrated in all 32 cases. In 4 of these the serological type had changed. The sensitivities to tetracycline corresponded to earlier sensitivities and where there had been a change it was regarded as falling within the limits of technical error.

Table 3 shows the percentual distribution of the penicillin and tetracycline sensitivities of all the 153 streptococci strains examined. All the strains can be taken as sensitive to penicillin, the majority (c. 90 per cent) of them at the borderline (1.5—3 µg/ml) for tetracycline. No significant sensitivity differences occurred between the groups treated with penicillin and tetracycline.

COMMENT

The results of the sensitivity determinations performed show that all the beta-haemolytic streptococci strains of the material must be considered sensitive, even highly sensitive to penicillin. Previously published reports (Forssell, Klemola) support this view. The re-appearances of streptococci at the follow-up may, however, be taken to prove that the concentration of DBED-penicillin was perhaps not, after all, sufficiently high. According to the literature (Stollermann Welch), an injection of 1 200 000 units of DBED penicillin produced 0.2—0.7 UI/ml concentrations in the blood for 10 days. As all the streptococci strains in question were sensitive in vitro to these concentrations, the penicillin concentrations in the tonsils and the blood obviously are not equally high. Assessed in this way, the penicillin concentration in the tonsils is less effective. Support for this view comes from the fact that streptococci were isolated in c. 35 per cent (Rhoads) from the tonsillary tissue despite the antibiotic therapy. As the patients returned home to their old infections environment the possibility of re-infection must also be taken into consideration in the three penicillin-treated

patients in whom no streptococci were demonstrated upon discharge from hospital. Allowing for this, however, it is still clear that DBED-penicillin administered upon discharge from hospital would have proved incapable of protecting the patients in question for two weeks.

The sensitivities of streptococci to 0.8—6 µg/ml of tetracycline, judging by in vitro examinations, must be regarded as weaker and none of the strains can be considered very sensitive. According to the literature, the mean tetracycline concentrations in the blood with a daily dose of 25 mg/kg (Friederiszick, Minsky) are for children c. 0.6—1 µg/ml with a daily dose of 1.0 g and for adults 1—3 µg/ml (Milberg, Putnam) administered perorally. It will be observed in the material of relapses after tetracycline therapy that all the strains were on the borderline of resistant or across it. It is consequently easy to understand that streptococci reappeared in as great a percentage of the cases as 50. The re-appearance rate in the tetracycline group was so high that it cannot be dismissed on the grounds of reinfection alone.

Although streptococci re-appeared in some of the cases after both DBED-penicillin and tetracycline therapy, it was possible to establish clearly that there was no increase in resistance and that the insufficient dosage did not cause increased resistance in vivo. The fact that with only a few exceptions the serological types remained unchanged indicate that the antibiotic therapy did apparently not change the streptococcal flora so as to remove the sensitive strains and leave the more resistant, earlier invisible strains.

Only by means of careful follow-ups can it be decided whether antibiotic therapy has been effective in scarlatinal tonsillitis. A bacterial examination immediately after the termination of therapy seems to be of minor significance. This is shown by Table 2 in which almost all the cases treated with tetracycline were streptococci-free when the administration of the drug was discontinued.

SUMMARY

The in vitro sensitivities of beta-haemolytic streptococci isolated at follow-up examinations from scarlet fever patients who had been treated with DBED-penicillin or tetracycline were studied.

In the penicillin group the sensitivity to penicillin before the treatment and at follow-up was 0.05 IU/ml and with one streptococcal strain 0.02 IU/ml or under with the other five. They could thus be considered sensitive *in vitro*. As streptococci re-appeared in only 6 per cent of the cases treated with penicillin and as 99 per cent of the 153 streptococci strains tested were sensitive to 0.05 IU/ml or less, it may be claimed that the sensitivity determination corresponds roughly to sensitivity *in vivo* but that in tonsillary tissue DBED-penicillin produces limit values which do not always give the desired effect. On the other hand no increase was observed in the resistance *in vivo*.

Tetracycline sensitivity determinations showed that all the streptococcal strains examined were either resistant or slightly resistant to tetracycline and that the *in vitro* result corresponded to that *in vivo* in which 50 per cent of the total material showed a re-appearance of streptococci after tetracycline therapy. Despite inadequate dosage no increase was observed in the resistance to tetracycline either.

The mean blood concentration values employed as controls in the resistance determinations were not fully acceptable as comparative data for assessing the effectiveness of penicillin in tonsillitis.

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FROM THE STATE SERUM INSTITUTE, HELSINKI

SPECIES-SPECIFIC PROPERTIES OF TISSUE CULTURE CELLS PROPAGATED IN DIFFERENT MEDIA

I

STUDIES IN GUINEA-PIG ANAPHYLAXIS

by

T. VAINIO¹ and A. LAHTI

(Received for publication September 1, 1958)

The view that the nature of the neoplastic cells depends essentially on the loss of antigenic specificities has attracted increasing attention (2, 4). There are few, if any, experimental data indicating which antigens are most profoundly influenced, but many experiments seem to suggest that a disturbance of the tissue- or/and organ-specific pattern is involved (10, 11, 16). Furthermore, the fact that neoplastic cells grow progressively in hosts reacting strongly against the corresponding normal cells seems to indicate that the »individual» antigens are of essential significance from this standpoint. That the strain- and species-specific antigens are not qualitatively affected is proved by experiments in which various neoplastic cells produced species-specific antibodies (8, 12), as well as in experiments where antibodies against different normal tissues were cytotoxic against, and reacted serologically with, neoplastic cells of the same species (3, 7, 8).

It may be suggested that malignant cells cultivated for a long period *in vitro* lose some of their original antigenic specificities, especially if cultivated in protein media of foreign origin. In the experiments described in what follows we tried to find out to what

¹ Aided by a grant from the Sigrid Jusélius Foundation.

extent species-specific, anaphylaxis-producing antigens are influenced in malignant cells cultivated for a long period and in benign cells cultivated over one passage. We tried to distinguish between the sensitizing and the anaphylaxis-producing effects of serum and tissue.

METHODS

The *antigenic doses* were prepared from the following materials:

- | | |
|---------------------------------------|---|
| Fresh active human serum | 1. HeLa cell homogenate (HeLa _{akt.}) |
| Partly denatured, 65°C heated hu- | Cells cultivated in active human |
| man serum | serum |
| Active rabbit serum | 2. HeLa cell homogenate (HeLa _{65°}). |
| Active calf serum | Cells cultivated in 65°C heated |
| Saline soluble fraction of the human | human serum |
| pulmonary homogenate (HPT) | |
| Saline soluble fraction of the human | 3. HeLa cell homogenate (HeLa _R). |
| pulmonary cancer homogenate | Cells cultivated in rabbit serum |
| (HCP) | |
| Saline soluble fraction of the rabbit | 4. HeLa cell homogenate (HeLa _C) |
| pulmonary homogenate (RPT) | Cells cultivated in calf serum |
| One passage human amniotic cells | |
| Continuous human amniotic line (CAL) | |

Normal and malignant pulmonary tissues were obtained from the same patient by operation. One passage amniotic and continuous amniotic cells were not prepared from the same placenta. The different HeLa lines had been maintained at the State Serum Institute for at least half an year.

The protein content of the different preparations was measured by the method of Weichselbaum and calculated by approximation.

The sensitizing doses were subcutaneously administered to guinea-pigs of middle size.

The challenge doses were injected intracardially 25—35 days later using concentrated preparations. Hereby we tried to avoid circulatory overloading.

Aware of the existing diversities of opinion concerning the most typical symptoms in guinea-pig anaphylaxis, we adopted the following criteria for the strength of the phenomenon:

- + slight scratching of the nose, coughing, gasping and bristling of hair on the back
- ++ pronounced scratching of the nose and coughing, mild convulsions, and weak symptoms of shock
- +++ severe convulsions, loss of balance, severe symptoms of shock
- ++++ death with anaphylactic symptoms (convulsions and jumping into the air, urination and defecation, prostration)

The rectal temperature of the guinea-pigs was measured before cardiac puncture and five, ten and twenty minutes afterwards. We found, however, that little benefit was derived from this determination, a definite fall in temperature being noted only in severe shock.

RESULTS

In the tests tabulated in Table 1, anaphylactic reactions occurred in guinea-pigs sensitized with fresh active or 65°C heated human serum pool. The table shows that in spite of the fact that the guinea-pigs reacted with pronounced anaphylactic symptoms after administration of rather small quantities of human serum protein, they did not show any reaction when the challenge dose contained homogenized HeLa cells in large quantities. By contrast, when the challenge dose contained amnion cell homogenate, the human serum sensitized guinea-pigs reacted strongly. As was to be expected, the same type of reaction occurred when the challenge dose contained antigens prepared from human pulmonary tissue or cancer tissue.

In Table 2, opposite tests are presented. Using as antigenic material HeLa cells cultivated in fresh active human serum or in human serum heated at 65°C it was possible to sensitize the guinea-pigs. The animals thus sensitized did not react when challenged with fresh human serum as antigenic material, but strong reactions were observed when material prepared from normal or malignant human tissues were used instead. It was not possible to confirm the weak reaction observed in case no. 58.

Since the same anaphylaxis-producing antigens had been demonstrated in HeLa cells and in normal and malignant tissue, it was interesting to repeat the tests in a contrary fashion. Therefore, guinea-pigs were sensitized with saline soluble fractions of

TABLE I

ANAPHYLACTIC REACTIONS OF GUINEA-PIGS SENSITIZED WITH FRESH ACTIVE HUMAN SERUM OR HUMAN SERUM HEATED AT 65°C.
THE ANTIGENIC MATERIAL HAS BEEN APPROXIMATELY MEASURED AS THE PROTEIN CONTENT OF THE SOLUTIONS IN QUESTION. HPT =
SALINE SOLUBLE PART OF HUMAN PULMONARY TISSUE HOMOGENATE. HPC = SALINE SOLUBLE PART OF HUMAN PULMONARY CANCER
HOMOGENATE. FOR OTHER EXPLANATION SEE TEXT

No. of Guinea-pig	Sensitizing Dose		Challenge Dose		Reaction	Antigenic Material	Amount of Protein	Controlling Dose		Reaction
	Antigenic Material	Amount of Protein	Antigenic Material	Amount of Protein				Antigenic Material	Amount of Protein	
1.	Human serumakt.	7 mg.	Human serumakt.	7 mg.	+++—					
2.	Human serumakt.	7 *	Human serumakt.	7 *	+++—					
3.	Human serumakt.	7 *	Human serumakt.	7 *	+++—					
4.	Human serumakt.	7 *	Human serumakt.	7 *	+++—					
5.	Human serumakt.	7 *	Human serumakt.	7 *	+++—					
6.	Human serumakt.	7 *	Human serum65°	3 *	+++—					
7.	Human serum65°	7 *	Human serum65°	1.5 *	+++—					
8.	Human serumakt.	7 *	HeLaAkt.	14 *	— — —	Human serumakt.				
9.	Human serumakt.	7 *	HeLaAkt.	7 *	— — —	Human serumakt.				
10.	Human serumakt.	7 *	HeLaAkt.	2 *	— — —	Human serumakt.				
11.	Human serumakt.	7 *	HeLa65°	7 *	— — —	Human serumakt.				
12.	Human serumakt.	7 *	HeLa65°	7 *	— — —	Human serumakt.				
13.	Human serumakt.	7 *	HeLa65°	7 *	— — —	Human serumakt.				
14.	Human serumakt.	7 *	Amnion cells	1.5 *	— — —					
15.	Human serumakt.	7 *	Amnion cells	30 *	— + —					
16.	Human serumakt.	7 *	Amnion cells	40 *	— + + —					
17.	Human serumakt.	7 *	Human serumakt.	14 *	— + + +					
18.	Human serumakt.	7 *	HeLa65°	4 *	— + — —					
19.	Human serumakt.	7 *	HPT	4 *	— + + +					
20.	Human serum65°	7 *	HPC	4 *	— + + +					
21.	Human serum65°	7 *	HPT	4 *	— + + +					
22.	Human serum65°	7 *	HPC	2 *	— + + +					

TABLE 2

ANAPHYLACTIC REACTIONS OF GUINEA-PIGS SENSITIZED WITH HOMOGENATES OF HELa CELLS PROPAGATED IN FRESH ACTIVE HUMAN SERUM OR HUMAN SERUM HEATED AT 65°C

No. of Guinea- pig	Sensitizing Dose		Challenge Dose		Reaction
	Antigenic Material	Amount of Protein	Antigenic Material	Amount of Protein	
47.	HeLa _{akt.}	7 mg.	HeLa _{akt.}	14 mg.	+++
48.	HeLa _{akt.}	7 "	HeLa _{akt.}	7 "	+++
49.	HeLa _{akt.}	7 "	HeLa _{65°}	10 "	---
50.	HeLa _{akt.}	7 "	HeLa _{65°}	7 "	+++
51.	HeLa _{akt.}	7 "	Human serum _{akt.}	7 "	---
52.	HeLa _{akt.}	7 "	Human serum _{akt.}	7 "	---
53.	HeLa _{akt.}	7 "	HPC	8 "	++++
54.	HeLa _{65°}	7 "	HeLa _{65°}	7 "	---
55.	HeLa _{65°}	7 "	HeLa _{65°}	10 "	---
56.	HeLa _{65°}	7 "	HeLa _{akt.}	12 "	+++
57.	HeLa _{65°}	7 "	HeLa _{akt.}	7 "	---
58.	HeLa _{65°}	7 "	Human serum _{akt.}	7 "	---
59.	HeLa _{65°}	7 "	Human serum _{akt.}	35 "	---
60.	HeLa _{65°}	7 "	HPT	4 "	+++

human pulmonary tissue and pulmonary cancer homogenates (Table 3). The animals thus sensitized reacted rather strongly when HeLa or amnion cells cultivated in human serum were used as a challenge dose.

It was striking that the CAL-cells (continuous amniotic line cells) did not behave in the same way although the protein content of the preparations used as challenge dose was relatively high and the sensitization of the animals was confirmed.

An important aspect of our problem was to find out whether HeLa cells grown in foreign media could incorporate foreign protein molecules in such a way that these proteins retained their original antigenic properties. For this purpose we sensitized guinea-pigs with rabbit and calf serum (Tables 4 and 5). When the challenge dose contained homologous antigens, the test animals reacted after administration of a relatively small amount of protein (one quarter of the sensitizing dose). None of them reacted when HeLa cells cultivated in calf or rabbit serum were used as challenge doses, even

TABLE 3

ANAPHYLACTIC REACTIONS OF GUINEA-PIGS SENSITIZED WITH SALINE SOLUBLE PART OF HUMAN PULMONARY TISSUE HOMOGENATE (HPT) OR OF HUMAN PULMONARY CANCER HOMOGENATE (HPC). CAL = CONTINUOUS AMNIOTIC LINE

No. of Guinea-pig	Sensitizing Dose		Challenge Dose		Reaction	Controlling Dose		Reaction
	Antigenic Material	Amount of Protein	Antigenic Material	Amount of Protein		Anti-genic Material	Amount of Protein	
61.	HPT	5 mg.	HeLa _{akt.}	22 mg.	++--			
62.	HPT	5 *	HeLa _{65°}	14 *	++-			
63.	HPT	5 *	CAL	6 *	-- --			
64.	HPT	5 *	CAL	20 *	-- --	HPC	4 mg.	+++
65.	HPT	5 *	Amnion cells	20 *	++--			
66.	HPT	5 *	HPC	4 *	++ +			
67.	HPC	4 *	HeLa _{akt.}	2 *	++ -			
68.	HPC	4 *	HeLa _{65°}	14 *	++ -			
69.	HPC	4 *	CAL	6 *	-- --			
70.	HPC	4 *	CAL	20 *	-- --	HPT	5 *	+++
71.	HPC	4 *	Amnion cells	20 *	++ -			
72.	HPC	4 *	HPC	4 *	++++			
73.	HPC	4 *	Amnion cells	20 *	++ +			

TABLE 4

REACTIONS OF GUINEA-PIGS SENSITIZED WITH FRESH ACTIVE RABBIT SERUM (7 MG). HELAR = HE LA LINE ADAPTED TO RABBIT SERUM MEDIA.
RPT = SALINE SOLUBLE PART OF RABBIT PULMONARY HOMOGENATE

No. of Guinea-pig	Challenge Dose		Reaction	Controlling Dose		Reaction
	Antigenic Material	Amount of Protein		Antigenic Material	Amount of Protein	
23.	Rabbit serum _{akt.}	7 mg.	++ +			
24.	Rabbit serum _{akt.}	7 *	++++			
25.	Rabbit serum _{56°}	7 *	++ +			
26.	Rabbit serum _{akt.}	3.5 *	++ +			
27.	Rabbit serum _{akt.}	2 *	++ +			
28.	Rabbit serum _{akt.}	9.7 *	++ +			
29.	HeLa _R	2 *	-- --			
30.	HeLa _R	2 *	-- --	Rabbit serum _{akt.}	7 mg.	++ +
31.	HeLa _R	7 *	-- --			
32.	HeLa _R	15 *	-- --			
33.	HeLa _R	25 *	-- --	Rabbit serum _{akt.}	3.5 mg.	++ +
34.	RPT	8 *	+ + -			
35.	RPT	4 *	+ + +			
36.	Human serum _{akt.}	7 *	-- --			
37.	Human serum _{akt.}	7 *	-- --	Rabbit serum _{akt.}	2 mg.	++ +
38.	Human serum _{akt.}	7 *	-- --			

TABLE 5

ANAPHYLACTIC REACTIONS OF GUINEA-PIGS SENSITIZED WITH FRESH ACTIVE CALF SERUM (7 MG). HELAC = HELA LINE ADAPTED TO CALF SERUM MEDIA

No. of Guineapig	Challenge Dose		Reaction	Controlling Dose		Reaction
	Antigenic Material	Amount of Protein		Antigenic Material	Amount of Protein	
39.	Calf serum _{akt.}	7 mg.	++++			
40.	Calf serum _{akt.}	7 *	++++			
41.	Calf serum _{akt.}	3 *	++++			
42.	Calf serum _{akt.}	2 *	+++			
43.	HeLa _C	20 *	---	Calf serum _{akt.}	1.5 mg.	---
44.	HeLa _C	14 *	---	Calf serum _{akt.}	1.5 *	+
45.	HeLa _{65°}	7 *	---	Calf serum _{akt.}	3 *	+
46.	Human serum _{akt.}	7 *	---	Calf serum _{akt.}	3 *	+

though the protein content was high. Both the HeLa_C cells and the HeLa_R cells were well adapted to foreign media and had been cultivated over several passages. No unspecific reaction were observed when calf or rabbit protein sensitized guinea-pigs were challenged with human serum.

DISCUSSION

In contrast to certain results indicating that the HeLa cells posses human properties (3, 9, 14) and cross-react with various human malignant tumors in culture (13), anaphylaxis-producing human antigens reacting like those of human serum were not demonstrated in our experiments. That this result does not prove the complete absence of human »markers» in the HeLa cells is shown by those tests where guinea-pigs sensitized with normal or malignant human tissue homogenates reacted strongly when challenged with HeLa material and vice versa. Furthermore, species-specific serum-like determinants may be present in tissue culture cells, also, as shown in the tests with one passage amnion cells. Even though such a conclusion is tempting, it cannot, on the basis of the few tissue culture materials used, be claimed that this lack of serum-like reactive species-specific patterns is typical of malignant cells

cultivated over several passages. In double diffusion precipitation tests similar differences between HeLa and amnion cells have been demonstrated (15), which seems to suggest that human species-specific proteins like those present in serum are lacking or occur only in small numbers in the HeLa cells.

On the basis of the experiments described in the foregoing it appears that HeLa cells growing in foreign media do not incorporate foreign protein molecules in such a way that these would retain their original antigenic properties. This is in keeping with the results of certain experiments performed using human malignant tumors grown in rats and tested by Ouchterlony's technique (5), but opposite results have been obtained when normal tissues have been cultivated in foreign media (6). The negative results of the present experiments corroborate the view that serum »contamination« of washed, trypsinized tissue culture material can be excluded. In view of the minute amount of serum protein which caused anaphylactic reactions in the guinea-pigs sensitized with rabbit or calf serum, and considering the complete unresponsiveness of the same animals after administration of large amounts of HeLa_R or HeLa_C materials, we seem to be justified in regarding our washing methods as adequate with regard to the abolishment of protein adsorbed from the growth medium.

In this connection we do not regard it as necessary to discuss the specificity of guinea-pig anaphylaxis and its mechanism. Concerning the former we refer the reader to the extensive experiments of Zilber (17), and the knowledge on the anaphylactic mechanism which has accumulated up to date is presented in a monograph by Becker (1).

SUMMARY

The question of whether the HeLa cells and amnion cells possess human species-specific properties was studied from the standpoint of guinea-pig anaphylaxis. The following conclusions were made:

HeLa cells propagated in human serum do not seem to contain the human species-specific antigens which occur in human serum. The amnion cells used seemed to contain some of these antigens.

Both the HeLa cells and the amnion cells used contained human species-specific antigens of the type observed in human pulmonary

tissue and cancer homogenates. These antigens were not observed in continuous amniotic cells, but the experiments performed were few.

HeLa cells propagated over several passages in rabbit or calf serum do not incorporate foreign antigenic material in such a way that this would cause an anaphylactic reaction in sensitized guinea-pigs.

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FROM THE STATE SERUM INSTITUTE, HELSINKI

SPECIES-SPECIFIC PROPERTIES OF TISSUE
CULTURE CELLS PROPAGATED IN DIFFERENT MEDIA

II

STUDIES USING THE GEL DIFFUSION PRECIPITATION AND GAMMA-
GLOBULIN INHIBITION TECHNIQUES

by

TAPANI VAINIO¹ and KARI PENTTINEN¹

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In previous investigations the nature of the antigens was studied in tissue culture cells which produce cytotoxic antibodies (6) and guinea-pig anaphylaxis (7). Various antisera against human antigenic materials were found to be cytotoxic to HeLa cells in tissue culture, which shows that these cells possess the same kind of antigens as normal human tissues and sera. On the other hand, it was not possible to demonstrate the anaphylaxis-producing antigens which are present in normal human serum and also in »benign» amnion cells in culture. Owing to the somewhat contradictory results obtained with regard to the presence of human serum-like reactive antigens in tissue culture cells it was suggested that the results probably were dependent on the technique employed. Therefore we decided to study the tissue culture antigens by several different serological techniques in order to determine which species-specific human antigens are present in tissue culture cells. In the present investigation the gel diffusion precipitation technique and anti-gammaglobulin inhibition tests were used.

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METHODS

Tissue Culture Technique. — The original HeLa cell strain used was received in 1955 from the Tuskegee Institute and has been maintained in continuous cultivation in 30 per cent human serum and 70 per cent Hanks solution. The serum used for this cell line was not inactivated, and this line is here called HeLa_{act}. Another HeLa cell line called HeLa_{65°} was cultivated in the same manner except that the serum used was heated at 65°C for half an hour before use. A third HeLa cell line, which in 1957 was obtained from the original line and adapted to grow in inactivated rabbit serum, is called HeLa_R. The subcultures have been repeated in Roux' bottles at 2—7 days' intervals. The cells were released from the glass by 0.5 per cent trypsin (Bacto 1/250) and dispersed by 0.1 per cent trypsin in a waterbath at 37°C for 30 minutes.

A continuous amniotic cell line (CAL) was received from Dr. T. Wesslén, Uppsala, in 1958 and has been maintained in exactly the same way as the HeLa strains, except that the serum used was heated at 56°C for half an hour and filtered before use. At the preparation of the subcultures the pH of the growth medium was adjusted to 7.4 with Hanks solution saturated with CO₂. When HeLa and CAL cells were used, the inoculum per bottle was 1 million cells.

Human amniotic cells were cultivated as follows: Placentae were obtained within twelve hours after delivery. All membranes which were detached or badly broken were discarded. The membranes were separated with instruments and weighed. Then they were treated at room temperature for 5 minutes in 100 ml of 0.25 per cent trypsin in phosphate-buffered saline. After discarding the trypsin, the membranes were gently shaken in 250 ml of phosphatebuffered saline. This was repeated three times. Any slime was detached with instruments as carefully as possible. The membranes were cut, 0.25 per cent trypsin in phosphate-buffered saline was added in an amount corresponding ten times to the weight of the membrane and the membranes were kept for one hour in a waterbath at 30°C. Then the trypsin was decanted, and the same amount was added for another hour. The membrane was gently shaken and the supernatant was discarded through gaze. Subsequently the membrane was shaken in 70 ml of two per cent

calf serum in phosphate-buffered saline. This was repeated, and the two supernatants were combined and centrifuged for 10 minutes at 800—1000 r.p.m. The supernatant was discarded and the cells were suspended in 20 ml of 20 per cent inactivated human serum and 80 per cent of the amino acid constituent of Parker's solution. The cells were counted after the suspension had been diluted one to four in 0.1 per cent trypan blue solution in saline. If the number of cells stained by trypan blue was below 10 per cent of the total count, the cells were approved for culture. This was usually the case when the procedure here described was used. In the Roux bottles an inoculum of 20 million cells was used, which within 2—5 days resulted in a coherent sheet of amniotic cells. As growth medium, 20 per cent inactivated human serum pool in the amino acid constituent of Parker's solution was used. The pH was adjusted at the outset as described above. Much smaller inocula may be used if the percentage of stained cells is very small and the cells in the counting chamber do not show any granulation. From one placenta as much as six Roux bottles, ready for use in a few days, may be prepared.

As will be mentioned later, after outgrowth of the cells the human serum pool was in some experiments replaced by inactivated horse serum.

All tissue culture fluids used contained 50 units of penicillin and 50 gammas of streptomycin per ml. To the amniotic cell cultures 25 units of mycostatin per ml were also added.

Antigenic Materials. — Tissue culture antigens were prepared from all the lines mentioned above. The subcultures of the HeLa lines were propagated in active human serum ($\text{HeLa}_{\text{act.}}$), in human serum heated at 65°C ($\text{HeLa}_{65^{\circ}}$), and in inactive rabbit serum (HeLa_R). The continuous human amniotic line (CAL) was maintained in inactive human serum, the amnion cells in active human serum or horse serum. For immunization freezed cells were quickly thawed and injected intravenously into rabbits. The protein content of the antigenic materials was measured by Weichselbaum's modification. A total of 150—200 mg of protein was injected into each rabbit in five doses at two days' intervals, and ten days after the last injection the serum was collected after cardiac puncture. Using the same method we prepared rabbit antisera against active human serum and human serum heated at 65°C and against super-

natants of human pulmonary tissue and cancer homogenates. For the tests part of the antigenic material was treated sonically, whilst the rest was homogenized in a glass grinder.

When preparing antigenic materials, the cells were mainly released from the glass by trypsin after which they were washed three times with phosphate-buffered saline. Differences between the behaviour of cell lines in question cannot depend on the loosely attached environmental proteins.

Double Diffusion Precipitation Tests. — were performed by the method of Ouchterlony (4). Sodium chloride in physiological concentration (0.9 per cent) was added to 2 per cent of purified agar, and the pH was adjusted to 8.2. After addition of merthiolate, agar was melted and placed in Petri dishes. A variety of different matrices were used, only two of which are shown in the following figures. After addition of antigen and antibody solution to the dishes in question, these were left for five days in a humid atmosphere at 37°C and for further three days at room temperature. Photographs of the dishes were taken with a black background and oblique light.

Anti-gammaglobulin Inhibition Tests (AHGGI). — These were based on the fact that human gammaglobulin is capable of preventing the anti-human gammaglobulin effect in tests performed by Coombs' technique. Different quantities of AHGG were absorbed at 37°C with two-fold titrated antigenic solutions. One hour later, D-homozygote human red cells loaded with incomplete anti-D antibodies were added to the mixture. The results were read after the mixture had been kept for ten minutes at room temperature. Control absorptions were carried out using serial dilutions of various normal human sera. In these as well as in the gel diffusion experiments sonic-treated cells were used to some extent.

RESULTS

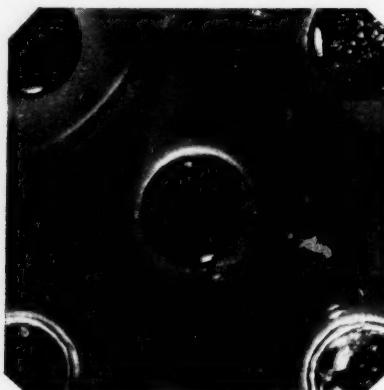
Double Diffusion Precipitation. — Although all the anti-HeLa sera used in this study were found to contain complement-fixing antibodies (Table 1), they failed to react with human serum diluted 1: 10 in the gel diffusion experiments. By contrast, anti-amnion serum reacted with human serum, forming two precipitation lines (Fig. 1). Two lines were also obtained in precipitation tests with

TABLE 1
OF TESTS SHOWING THE ENDPOINTS OF TWO-FOLD TITRATED ANTISERA

Antisera	Antigens				
	Human Serum 7 mg/ml	Amnion Cells 10 mg/ml	HeLaact. Cells 20 mg/ml	CAL Cells 6 mg/ml	Sal.
Anti-HeLa _{act.}	1/2	1/8	1/256	1/4	—
Anti-HeLa _{65°}	1/1	1/4	1/512	1/8	—
Anti-HeLa _R	1/2	1/16	1/128	1/4	—

In the center: anti-amnion serum

Human serum
(7 mg/ml)



HeLa_{65°}(15 mg/ml)
sonic-treated

HeLa_{act.}
(10 mg/ml)
sonic-treated

CAL (10 mg/ml)
sonic-treated

Fig. 1. — The strength of the antigenic solutions are expressed as amount of protein per ml.

In the center: anti-amnion serum

amnion cells
(20 mg/ml)

HeLa_{65°}(15 mg/ml)
sonic-treated

saline

Fig. 2.

In the center: anti-HeLa_{65°}

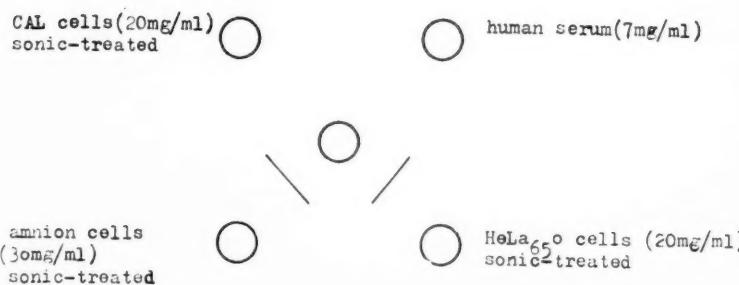


Fig. 3. — Attempts to photograph the weak lines were unsuccessful

In the center: anti-human serum_{65°}



Fig. 4.

anti-amnion serum and homologous antigens (Fig. 2). The anti-HeLa sera were definitely poor in precipitins, only one of them reacting with sonic-treated HeLa and amnion cells (Fig. 3). In the reverse experiments the antisera against human serum as well as the sera against human pulmonary tissue and cancer failed to precipitate the HeLa and CAL antigens, whilst they precipitated amnion cell antigens again forming two lines (Fig. 4 and 5). Sonic treatment of the cells did not seem to strengthen the results obtained.

In the center: anti-human serum_{65°}

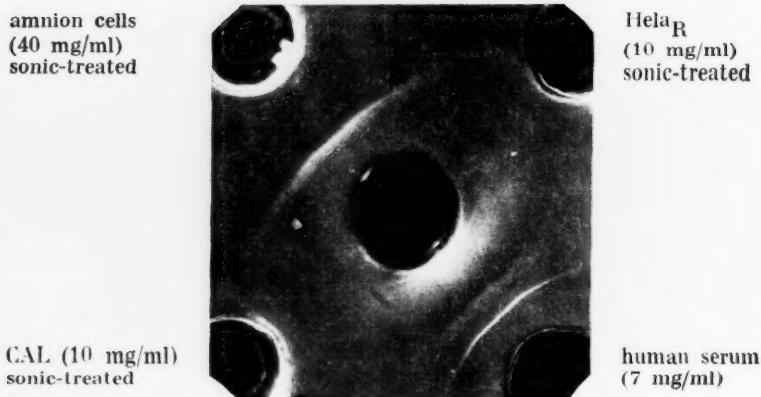


Fig. 5.

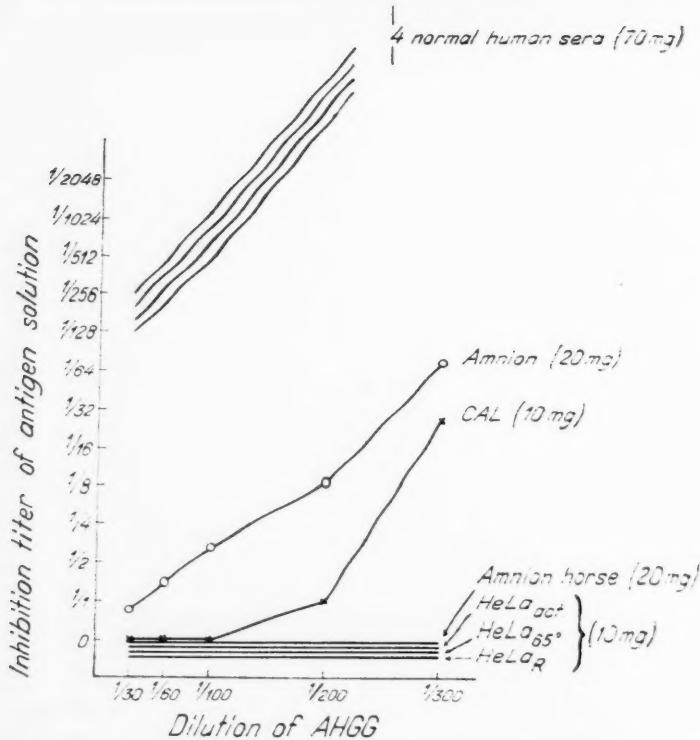


Fig. 6. — Inhibition titres of the antigenic solutions against different concentrations of anti-human gammaglobulin measured by Coombs' technique. The protein concentrations of the antigenic solutions before titration are indicated in brackets as mg per ml.

It was repeatedly observed that when amnion cells were cultivated in medium containing horse serum, the cells completely lost their capacity of reacting with anti-sera against human serum in gel diffusion tests.

When antisera against human serum were absorbed with HeLa antigens, the reaction of these antisera to homologous antigens was not changed. This result was confirmed using Björklund's inhibition plates (2).

Anti-gammaglobulin Inhibition Tests. — AHGGI-tests were performed with both sonic-treated and freezed-thawn tissue culture materials. The results of these tests, shown in fig. 6, resemble closely those obtained in the gel diffusion experiments. Our HeLa lines did not seem to contain material capable of inhibiting the AHGG effect. The CAL cells had a certain inhibitory effect on a low concentration of AHGG. The inhibitory effect of the amnion cells was even stronger than that of the CAL cells, although definitely weaker than the inhibitory effect of normal human serum.

As also appears in fig. 6, the entire AHGGI activity of the amnion cells seemed to disappear when the cells were cultivated in medium containing horse serum.

DISCUSSION

Human species-specific antigens were hardly demonstrable in the HeLa cells using the techniques here described. The only evidence of species-specificity in the HeLa cells was the weak cross-reaction observed between amnion antigens and one of the anti-HeLa sera. No cross-reaction occurred between the anti-HeLa sera and human serum nor between the anti-sera against human serum and the HeLa antigens. This seems to indicate that *the line of HeLa cells used does not contain human antigens of the type present in normal serum*. This conclusion is corroborated by our earlier results on anaphylaxis-producing antigens (7), but it cannot be postulated that the same conclusion would be justified if another serological technique had been used. In CF tests our anti-HeLa sera as well as our anti-amnion sera and anti-serum against human serum showed antibodies against different human antigens of several sera, normal tissues, cancer tissues and human cells in culture (8). Furthermore, the antisera against different human

antigens were cytotoxic to the HeLa cells (6). On the basis of these observations we cannot postulate that human cancer cells in culture are not immunologically human. We only wish to draw attention to the fact that no serological technique alone yields more than a limited answer concerning tissue culture immunology.

On the basis of the AHGGI tests it is possible to make certain quantitative approximations. As seen in Fig. 6, about 1 mg of amnion cell protein has the same inhibitory effect on AHGG as normal human serum diluted 1: 5000 (0.012—0.014 mg protein). This means that only a part of amnion protein is capable of inhibiting AHGG, or that a total of 1 per cent of the amnion cell protein is functioning in inhibition tests as gammaglobulin. This small amount of antigenic gammaglobulin seems completely to disappear when amnion cells are cultivated in foreign protein medium. It will be interesting to see whether the foreign gammaglobulin in the amnion cells is also measurable. This would reveal in which way »benign» tissue culture cells bind environmental globulins. Experiments concerning this point are in progress.

The question of how cultivated cells use protein is little investigated and poorly understood. All fractions of the protein solution seem to disappear equally during cultivation (1), but how the proteins are bound is not clear. Certain data seem to suggest that malignant cells do not bind the globulins of the growth medium because they lack some cytoplasmic protein which forms complexes with the globulins (5). That important changes in the cytoplasmic proteins occur during carcinogenesis has been shown by Miller *et al.* (3). Furthermore, we know that the organspecific patterns are changed when cells turn malignant in tissue culture (9).

On the basis of the results reported in the foregoing it may be suggested that normal cells bind the environmental globulins into some special factor which is lacking in the HeLa cells or that the HeLa cells destroy species-specific patterns of human protein. Whether this behaviour is typical for all malignant cells cannot be postulated on the basis of the material used, especially not considering that in the AHGGI tests the CAL cells behaved more or less like amnion cells, although we have regarded the CAL cells as malignant. On the other hand, the carcinogenesis of the HeLa cells and the CAL cells is probably different, since the former are obtained from a malignant tumour and the latter have

turned «malignant» *in vitro*. This should be borne in mind when the capacity of the CAL cells of binding environmental proteins is assessed.

At one of the Symposia of the VII Congress of Microbiology, Ouchterlony presented a plate showing not only that his HeLa line reacted clearly like certain human serum antigens, but also that when cultivated in foreign serum medium its antiserum cross-reacted even with foreign serum antigens. In the light of the experiments reported here this result seems confusing and shows the importance of cautiousness in drawing conclusions even with regard to the antigenicity of the same line in different laboratories.

SUMMARY

The antigenicity of the HeLa cells was compared with that of continuous amniotic cells and amnion cells using the double diffusion precipitation and anti-gammaglobulin inhibition techniques. The results seem to indicate that HeLa cells cultivated in active human serum or in human serum heated at 65°C or in inactivated rabbit serum do not contain the antigens which are demonstrable in human serum. By contrast the amnion cells and the continuous amniotic cells contained those antigens. These human serum-like reactive antigens seemed to disappear from the amnion cells when they were cultivated in foreign serum.

The results are discussed in the light of previous experiments, and it is suggested that the HeLa cells are lacking some special factor which binds environmental globulins or that the HeLa cells destroy the species-specific patterns of human globulins.

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FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF TURKU

ACTIVITY OF SOME ENZYMES IN THE HYPOTHALAMO-
NEUROHYPOPHYSAL SYSTEM IN EXPERIMENTAL
DEHYDRATION AND REHYDRATION

A HISTOLOGICAL STUDY

by

ERKKI KIVALO,¹ URPO K. RINNE¹ and SIMO MÄKELÄ

(Received for publication August 27, 1958)

In the nucleus supraopticus (n.s.o.) and nucleus paraventricularis (n.p.v.) of the hypothalamus acetylcholinesterase (1), acid phosphatase (4) and succinic dehydrogenase (15) have been established histochemically, among others. In the neural lobe of the pituitary, too, the two last mentioned enzymes have been observed, although in considerably lesser quantity (9). On the other hand the conception is already prevalent according to which the cells of the above-mentioned nuclei produce the antidiuretic and oxytocin hormones, which migrate within the nerve fibres into the neurohypophysis (7). Several workers have been able to show a correlation between the histologically demonstrable Gomori substance and the changes in secretion of antidiuretic hormone produced by dehydration and rehydration (7, 6). In a previous investigation, the authors have found that acid phosphatase increases in the magnocellular hypothalamic nuclei under the effect of dehydration (13). In order to throw further light on the question, we undertook a study in which the effect of dehydration and rehydration upon the acetylcholinesterase and succinic dehydro-

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enase activity in the magnocellular nuclei of the hypothalamus was examined. In addition to these enzymes also the acid phosphatase was studied in the posterior lobe of the hypophysis.

MATERIAL AND METHODS

The investigation was carried out with altogether 56 adult female rats, half of them serving as a control series. The test animals were subjected to thirst during eight days, only dry food being given during this time. Part of the animals were then exterminated, while the rest of the test animals received an adequate quantity of water and were killed after two and five days of rehydration, respectively. The controls received water and food *ad libitum* throughout the entire test and were killed at the same times as the test animals. From the n.s.o. and n.p.v. of the test animals as well as of the controls and from the posterior lobe of their hypophysis the acetylcholinesterase was determinated according to Koelle's modification (8) and the succinic dehydrogenase according to Seligman and Rutenberg (14). Furthermore, with part of the animals, the acid phosphatase was determined from the posterior hypophysis according to Eränkö's modification (3). For the sake of control, the number of pituicyte mitoses was counted from hemalum-stained serial cuts of the posterior lobe.

RESULTS AND DISCUSSION

The acetylcholinesterase and succinic dehydrogenase activity in the n.s.o. and n.p.v. of the control animals was found to conform with previous results. Upon dehydration the activity of the said enzymes was clearly increased in both nuclei (Figs. 1, 2, 5, 6). After two days' rehydration no significant difference was observed any more with regard to succinic dehydrogenase between the animals of test and control series, whereas the acetylcholinesterase activity was still clearly increased with the test animals (Figs. 3 and 4). After five days' rehydration there was no difference with the controls in regard to the acetylcholinesterase activity either. — In the posterior lobe of the hypophysis no distinct acetylcholinesterase activity was observed with the controls nor with the test animals. On the other hand a slight acid phosphatase and succinic

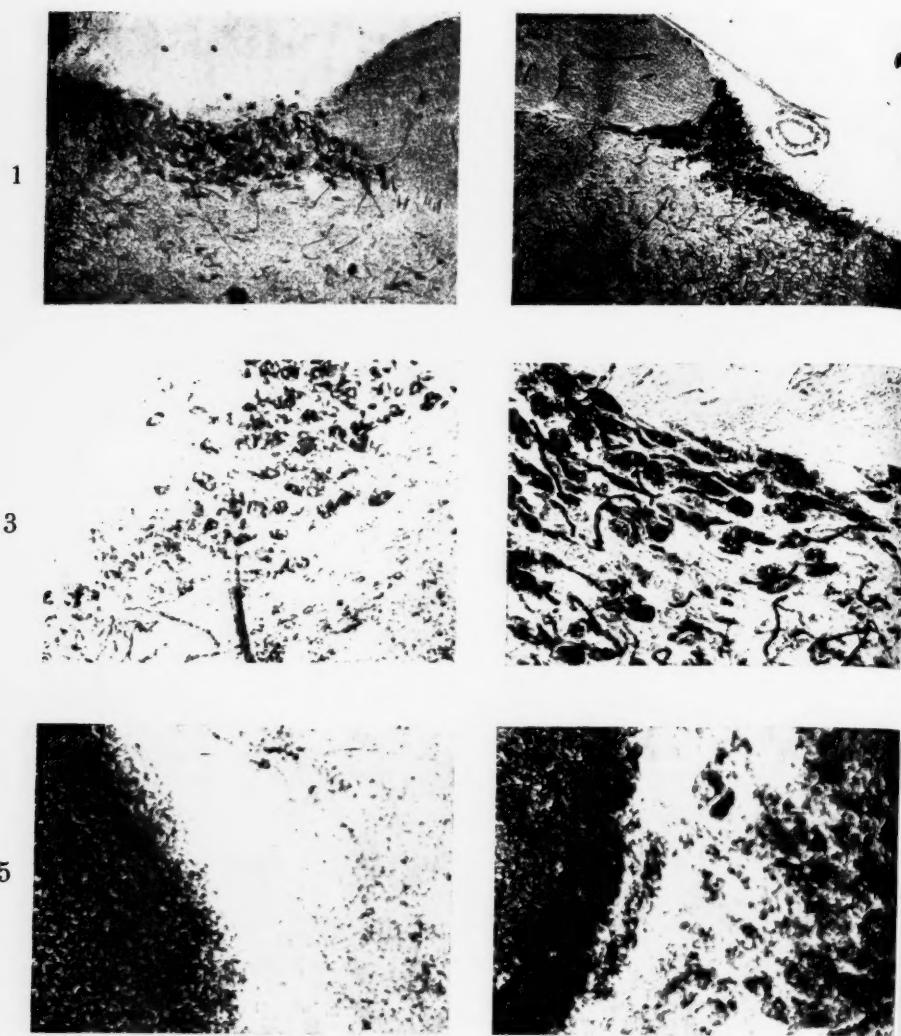


Fig. 1. — The activity of acetylcholinesterase in the nucleus supraopticus of a control rat. $\times 72$.

Fig. 2. — The activity of acetylcholinesterase in the nucleus supraopticus of a 8 days dehydrated rat. $\times 72$.

Fig. 3. — The activity of acetylcholinesterase in the nucleus supraopticus of a control rat. $\times 180$.

Fig. 4. — The activity of acetylcholinesterase in the nucleus supraopticus of a 2 days rehydrated rat. $\times 180$.

Fig. 5. — The activity of succinic dehydrogenase in the nucleus supraopticus of a control rat. $\times 180$.

Fig. 6. — The activity of succinic dehydrogenase in the nucleus supraopticus of a 8 days dehydrated rat. $\times 180$.

dehydrogenase activity was observed, although this was equal in the test animals and in those of the control series. In the controls no mitoses of the pituicytes in the posterior lobe of the hypophysis were observed, whereas the dehydrated animals displayed on an average 35 mitoses in one posterior lobe.

The observations indicate that sufficiently strong stimulation, in this case dehydration, causes an increase of the acetylcholinesterase and succinic dehydrogenase activity in the magnocellular hypothalamic nuclei. The same is also indicated in regard to acetylcholinesterase by the results of Pepler and Pearse (11) on stimulation with hypertonic NaCl. Moreover, the present result is in agreement with our previous findings (13) according to which dehydration causes increased acid phosphatase activity in the said nuclei. The result obtained here appears logical in respect to acetylcholinesterase inasmuch as the acetylcholine is generally assumed to be the chemical transmitter in the said nuclei responsible for stimulation (2, 12), more acetylcholinesterase being required to decompose its increased quantity. Obviously the stimulus employed was so strong that also the activity of the respiratory enzyme, the succinic dehydrogenase has increased on account of its effect, although the signification of this enzyme in the function of the nuclei has been found to be quite slight (16). In earlier investigations dehydration has been noted to produce mitoses in the pituicytes of the posterior lobe of the hypophysis, for which reason these have been assumed to be concerned in the mechanism of releasing neurosecretory substance from the nerve endings into the blood (10). Although dehydration produced mitoses, it did not cause any changes in the activity of the investigated enzymes in the posterior lobe of the hypophysis, thus suggesting that the function of the cells has not been accelerated. The occurrence of mitoses may possibly be explained by the suggestion that dehydration promotes cell division in general (5).

SUMMARY

The effect of dehydration and rehydration upon the acetylcholinesterase and succinic dehydrogenase activity in the hypothalamic magnocellular nuclei and in the posterior pituitary has been investigated with rats as experimental animals. In the posterior

lobe of the hypophysis also the acid phosphatase and mitoses of the pituicytes has been studied. After eight days' dehydration the activity of the said enzymes was significant increased in the n.s.o. and n.p.v. The succinic dehydrogenase activity was normal after two days' and the acetylcholinesterase activity after five days' rehydration. In the posterior pituitary no distinct difference was observed with respect to the investigated enzymes between the test and control animals. In the controls no pituitary mitoses was observed, whereas after dehydration there was about 35 mitoses in one posterior lobe. The significance of the investigated enzymes in the function of the n.s.o. and n.p.v. and the importance of the pituicytes in the mechanism of releasing neurosecretory substance into the blood is discussed.

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FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF TURKU

ALDEHYDE-FUCHSIN-POSITIVE MATERIAL IN SURGICAL BRAIN LESIONS

by

E. KIVALO and J. TYRKKÖ

(Received for publication December 11, 1958)

Aldehyde-fuchsin (AF) and chrome alum hematoxylin-phloxin (CAH) possess many similar staining properties. AF stains elastic fibres, mast-cell granules, gastric chief cells, beta cells of pancreatic islets and certain of the hypophyseal beta granules violet to purple. Alpha granules of the hypophysis stain orange red, delta granules green to greenish blue and chromophobe cells present a pale grey-green cytoplasm (7). With the CAH procedure the beta cells of the pancreatic islets are blue, alpha cells red; in the hypophysis, alpha cells are pink, beta cells grey-blue, and they are not readily distinguished from chromophobes (8). Both procedures have also been proved to be selective in the histological demonstration of the so-called neurosecretory material. Bargmann *et al.* (3) showed this in regard to the CAH procedure and Halmi and Davies (11) applied the AF method with preceding oxidation with a solution of acid permanganate, the neurosecretory material staining more clearly, by this procedure.

On the other hand, de Groot (10) found some CAH-positive material around electrically induced brain lesions. It was thought interesting to study the result of AF staining in about the same conditions.

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MATERIAL AND METHODS

Twenty-six adult female albino rats and one adult male albino rat, weighing about 200 g., were used as experimental animals. The animals were at first anaesthetized with ether, after which the skull was pierced with a dentist's drill. Lesions were induced in certain regions of the brain stem with a dissecting knife in one case and with a rotating blade attached to a dentist's drill in the other instances.

The lesions, one-sided in each single case, were induced in the following places:

1. The olfactory bulb of five animals was destroyed.
2. In five animals a deep, transversal lesion was produced in the frontal lobe.
3. In three animals lesion of the parietal lobe was produced.
4. In six animals a lesion was induced to the cerebellum.

Furthermore, the present experiment included five controls, on which a fictitious operation was performed by opening the scalp and breaking the exterior periost of the osseous cranium. Two animals bearing parietal lesions died of the shock subsequent upon the operation. One animal died of excessive depth of anaesthesia.

After the operation the animals were fed under normal diet. They were killed by rapid decapitation when 30 days had elapsed since the operation, slight anaesthesia by chloroform being applied prior to this.

The brains were removed, fixed in Bouin's solution for several weeks and then embedded in paraffin. Sections of $7\text{ }\mu$ were stained by the AF procedure after KMnO_4 oxidation (6).

RESULTS

The awakening after the operation lasted 15 to 30 minutes. In several instances the animals then showed initially, a strong flexion of body and extremities, succeeded by tonic extension after a short period. This ended in clonic convulsions which could last for several minutes. On the whole the fit was greatly similar to the epileptic grand-mal attack. Dyspnoea was frequently present, in which case artificial respiration and air applied at over-pressure were administered.

The animals bearing parietal lesion showed paralysis of the opposite pair of extremities for some time, while a lesion in the cerebellum resulted in transient hemiplegia. During about one week the experimental animals were unwilling to move or eat, the latter behaviour being particularly marked in the animals bearing a lesion of the olfactory bulb. For this reason these animals were at first fed with food very rich in water. Generally, their affectivity was markedly reduced; animals bearing lesions in the frontal lobe presented clearly increased dullness.

In the histological preparations the place of the lesion could be seen quite easily (Fig. 1 and 2). In most cases there was a perfora-



Fig. 1.

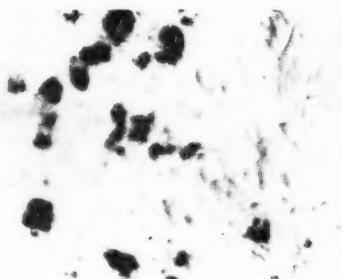


Fig. 2.

Fig. 1. — A photomicrograph of the parietal lobe of the rat brain. In the middle of the lesion, at margins of which there are some aldehyde-fuchsin-positive granules and pigment accumulations. $\times 120$.

Fig. 2. — A photomicrograph of the frontal lobe of the rat brain. The margin of the perforation is on the right, showing some aldehyde-fuchsin-positive granules, fibres and pigment accumulations. $\times 740$.

tion surrounded by fibrous scar tissue. AF-positive material occurred as granules of various size and shape, partially intracellularly but also outside of the cells. Pigment accumulations and/or cells were generally located in relatively large amounts at the very margins of the hole, whereas they could not be seen in the deeper tissue. In addition, there were also some AF-positive fibres in the scar tissue.

DISCUSSION

In the fibrous base tissue of a scar following a lesion of brain tissue there are also other cell elements in addition to blood pigment cells: fatty granula cells (degenerating Hortega-cells), fibro-

cytes and macrophages. Also inflammatory cells may be seen in a residual scar. However, these are limited to some few lymphocytes and plasma cells (13).

Mazzi (14), as well as Green and van Breemen (9), found that phagocytes and degenerating nervous elements may stain with the CAH procedure. On the other hand, according to Bangle (2), AF stains the following elements: 1) specific proteins, 2) specific mucopolysaccharides and 3) tissue aldehydes (from lipids). Many other authors have also reported that AF has an affinity to the sulfate esters of polysaccharides and in general to sulfonic groups (1, 4, 5, 15) and to aldehyde groups (4, 8, 11, 15), and in addition to this to some acid, probably COOH groups (12). Thus, *e.g.* macrophages, upon phagocytizing degenerating nervous tissues, may completely imitate the neurosecretory material in a histological preparation. Because of the non-specificity and broad spectrum of the employed staining procedure it seems difficult to differentiate the nature of the granulation surrounding the brain lesions. It is conceivable that by inducing lesions *e.g.* to the area of the tractus supraoptico-hypophysealis an accumulation of some AF-positive granulation around the brain lesion can be caused to occur, the differentiation of which from the neurosecretory material could meet with difficulties in this respect.

SUMMARY

Some AF-positive granulation was found surrounding lesions induced in various places of the brain tissue. Various staining possibilities of AF are discussed. The granulation is greatly resemblant of the so-called neurosecretory material but it is hardly a part of the latter. However, differentiation of the neurosecretory material from this pathological AF-positive granulation may meet with difficulties if the lesion is located in areas where neurosecretory material occurs naturally.

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FROM THE INSTITUTE OF ANATOMY, UNIVERSITY OF HELSINKI, AND THE
RESEARCH LABORATORIES OF THE STATE ALCOHOL MONOPOLY, HELSINKI

OCCURRENCE OF TRICHOSOMOIDES CRASSICAUDA
AMONG ALBINO LABORATORY RATS IN HELSINKI

by

JOHAN AHLQVIST and JUHANI KOHONEN

(Received for publication September 11, 1958)

During a preliminary investigation on the epithelium of the urinary bladder in rats, a parasite was observed in the lumen of the bladder, which later was found to be *Trichosomoides crassicauda* (*Nematoda, Trichinellidae*). This nematode had not previously been observed in Finland.

Laboratory rats were examined from five institutes in Helsinki in which rats are used for medical and biological experimental investigations. Parasites were found in the bladders of rats from all these institutes. A total number of 30 rats have been examined, and 16 of these had *T. crassicauda* in their bladders.

During its life cycle in the rat, this nematode may cause various pathological alterations, and even the death of the host. It therefore seems worth while to describe in brief its life cycle, and the pathological changes which it may cause in the host organism.

Trichosomoides crassicauda belongs to the same family, *Trichinellidae*, as the better known *Trichinella spiralis* of the class *Nematoda*. It occurs in the bladders of wild rats, especially, *Rattus norvegicus*, and was first described by Bellingham (1) in 1840. The male is much smaller than the female, and is often found parasitic in the vagina or uterus of the latter. According to Hall (2), the female is 10.5 to 13 mm long, and is about 200 μ thick in the posterior portion of the body. The body is covered by a chitinous cuticle.

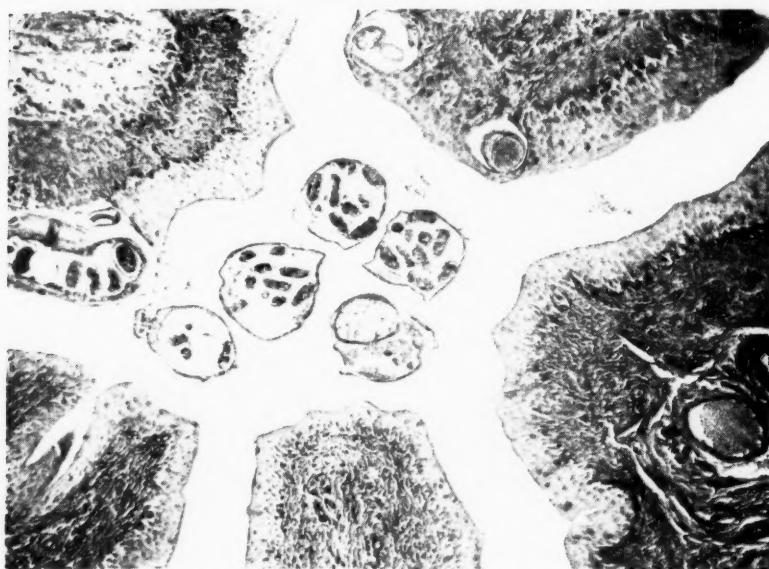


Fig. 1. — Photomicrograph of *Trichosomoides crassicauda*. Transverse section of a female located in the urinary bladder of a laboratory rat. $\times 70$. PAS-stain.

This can be seen encircling the digestive tract and sex organs in microscopical sections of the rat bladder (Fig. 1.).

Some disagreement exists concerning the life cycle of this parasite. The eggs containing embryos are passed in the urine of the host. These are able to live outside the host for only a short time, and are probably soon ingested by another rat together with food or water. After having been ingested by the rat, the egg shell opens and the larvae bore themselves into the wall of the intestine. The first experimental infestations were carried out by Yokogawa (5), who found larvae in the pleural and abdominal cavities and in the lungs of infected animals. From these experiments, he concluded that the larvae migrate through the body tissues and cavities to the lungs. Thomas (4) and others find it more probable that they bore themselves into the blood vessels of the intestinal wall, and reach the heart by way of the portal system. In the lungs they may reach the pleural cavity or the bronchi, or enter the blood vessels on the other side of the capillary bed. They circulate freely in the blood of the host, and are distributed to all parts of the body. In any case, probably only those that reach the kidneys find condi-

tions favorable for their further development. In the kidneys, they leave the blood stream to migrate to the pelvis of the kidney. Copulation takes place at any point in the urinary tract. The fertilized female usually passes to the bladder. The life cycle may be completed in fifteen days (4).

During their migration through the tissues of the host, the parasites may cause pathological alterations in various organs. Bleeding points have been observed in the lungs, liver and kidneys. The parasites may be seen alongside small blood vessels, with small haemorrhages beside them. The renal tubules have been found filled with coagulated blood. In some experiments (4), the animals have died a few days after the ingestion of eggs, showing evidence of pulmonary trouble. Amongst others, Löwenstein (3) during investigations on tumours of the rat urinary bladder, has observed papillomas often associated with *T. crassicauda*. Worms have been found imbedded in these tumours (4). Löwenstein (3) suggests that these papillomas might be caused by mechanical stimulation, or by some toxin secreted by the worm. Some of the animals of Thomas (4) remained dwarfed in size, their coats were roughened, and they were in a general anemic condition. The present authors have observed an infiltration of cells in the bladder epithelium, which in some respects bear a resemblance to mast cells, but in other respects differ from them. These cells seem to be associated with the occurrence of *T. crassicauda*. A separate report will be made on this.

According to the above mentioned facts, various pathological alterations caused by *T. crassicauda* may disturb experimental investigations on rats. As albino rats belonging to Wistar and Sprague-Dawley strains infected with *T. crassicauda* were found among the stocks of all the five laboratories investigated, it seems probable that they may also be found in other institutes. As stated, this nematode has not formerly been described in Finland but as it is common among wild rats in many European countries it may also be so here. The laboratory stocks may thus have been infected by wild rats in some laboratory, or possibly at some commercial breeding centre. It seems worth while to try to get rid of these parasites in those laboratories which use rats as experimental animals.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF
HELSINKI

BODY TEMPERATURE STUDIES ON A UNIFORM MICE-STRAIN

by

ALDUR W. ERIKSSON and OLE WASZ-HÖCKERT

(Received for publication September 24, 1958)

It was noted in connection with an investigation of the effect of chlorpromazine-hibernation on staphylococcal infection in mice (10) that the fairly large literature on artificial hibernation with mice as test animals contains nothing on intra- and inter-individual variations of body temperature in normal mice during certain degrees of activity. We found such information essential to our further studies. This and the fact that biochemical individuality and variation have been increasingly studied in recent years (11) prompted the present investigation.

MATERIAL

The study was based on a highly uniform strain of mice. We used Swiss Albino Webster mice, adult males with a weight range of 20—33 g and not earlier treated with chlorpromazine (5). The parents of the 30 mice used in the experiments were strongly inbred. The breed was imported from New City, New Jersey, U.S.A.

All the mice were kept in similar conditions, 5 to a cage. They were given water ad libitum and well-balanced food pellets (1). The room temperature was maintained at 17—19°C. The animals

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were adapted to laboratory conditions for one week before the beginning of the experiment. Each mouse was marked for recognition purposes. The same 30 mice were used for all the experiments.

TECHNIQUE

The temperature of the test animals was registered in normal physiological conditions, under stress and during hibernation.

The *temperature* was read with an electrical precision thermometer apparatus manufactured by Electrolaboratoriet, Copenhagen, with applicators of rectal type RM 4, diametre 2 mm, and needle type K, diametre 0.7 mm, shaped like an ordinary injection needle. The relevant technical details have been reported previously (10). In this investigation, all the rectal measurements were made at a depth of 20 mm in the sigmoideum for 10 seconds. The short time was to obviate stress effect on the body temperature of the animals.

The *stress effect* was studied by poking the needle applicator of the thermometer into the inguinal region of the mice for 20 seconds. This was taken as a major stress. The post-stress subcutaneous and rectal temperature and the behaviour of the animals was observed. The temperature, rectal and subcutaneous simultaneously, was read 10 seconds after the stress to exclude possible local hyperemia in the rectum etc. Injections were also considered a major stress. 4—5 days' rest was allowed between a major stress and hibernation. Animals which had not earlier suffered a stress were also used in the hibernation experiment, as controls.

To induce *hibernation* we used a 0.1% sterile, freshly-made, light-protected solution of chlorpromazine-hydrochloride (Largactil May & Baker). 30 mg/kg body weight of this solution was injected subcutaneously into the inguinal fold of the mice using a specially careful technique to avoid possible return oozing of the solution. Relative to the size of the test animals, the amount of fluid was rather large (0.6—1.0 ml). This was considered necessary, however, in order to prevent dehydration since the active fluid intake of a hibernating animal is almost nil for 12—24 hours. It should be noted, too, that higher chlorpromazine concentrations than 0.15% have a considerable irritant action, stress (6).

RESULTS

The following points were studied: the intra- and inter-individual variations in the diurnal rhythm of the body temperature, the temperature changes following stress and chlorpromazine hibernation.

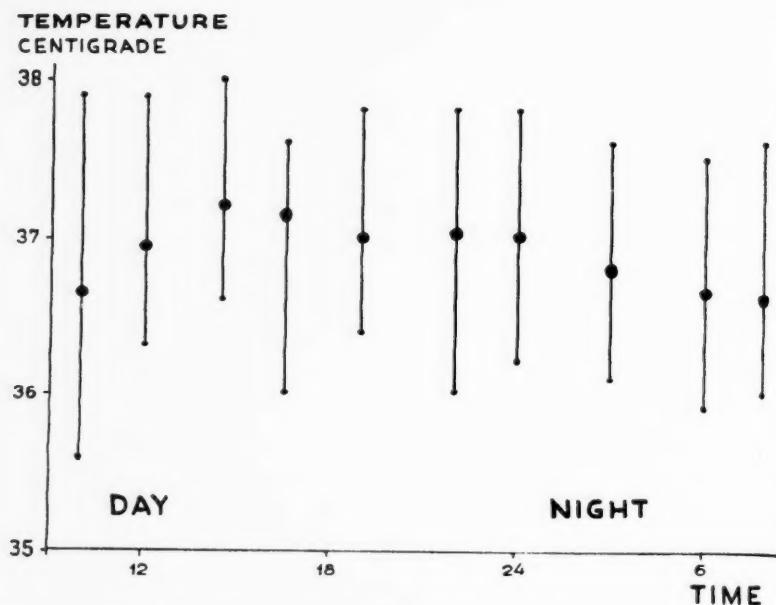


Fig. 1. — The diurnal rhythm and range of the rectal body temperature of 30 adult Swiss Albino Webster male mice.

Diurnal Rhythm. — It will be seen from *Fig. 1* that the rectal temperature mean variation range was 36.6—37.2° centigrade, with the peak at 1500 hours and the low at 0600 hours. The range of the 30 mice is also given in the figure. This, however, gives the so-to-say inter-individual differences. It was found from separate measurements that the *intra-individual* variations were also considerable. For instance, an animal with an extremely low temperature at 0600 could have an extremely high reading at 1600. This *intra-individual* variation we found to be correlated with the individual activity of the mice concerned. The same observation was made in the different cages. Thus the mean temperature

variation of 5 mice in one cage could vary according to the activity in the cage (especially if there was fighting).

Temperature Variations Following Stress. — In this experiment the temperature of the test animal was measured after »major stress», *i.e.* 20 seconds of poking with the needle applicator. The temperature was read 10 seconds after this stress for 20—50 minutes, every second-third minute. 30 mice were used although the temperature curves of only 8 animals are given in *Fig. 2*. These

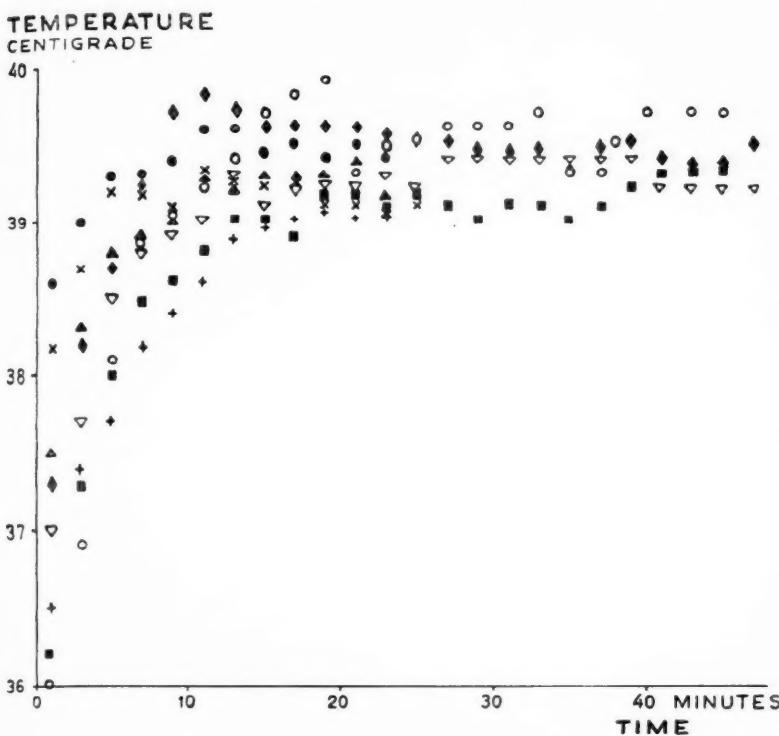


Fig. 2. — The effect of major stress — produced by poking with an injection needle — on the body temperature of 8 representative male mice.

curves were representative of the entire group. As can be seen from *Fig. 2*, the trend following stress is distinct, a rise of 1—4°C after 15 minutes. The range for the 30 mice is quite narrow after 15—20 minutes. In not one of the animals was a rapid fall in temperature registered after stress, though inter-individual differences were seen. By repeated major stresses in a test animal group it

was, however, possible to trace individual tendencies. For instance, »the kings of the cage» almost always had a higher and faster rise in body temperature than the weak and feeble mice. There was no definite correlation between body weight and temperature rise.

With the mice left in complete peace it took 10—40 minutes (as a rule longer for the »kings») for the temperature to return to normal. The results showed that the laboratory mice used here reacted to a pain stress with a considerable rise in temperature. The minor stress caused by handling the mice and taking the rectal temperature also caused a slight rise in temperature. The curve indicating the trend was flatter. This aspect has been dealt with in more detail in another paper (4).

Hibernation Curve. — Fig. 3 shows that with the amount of the

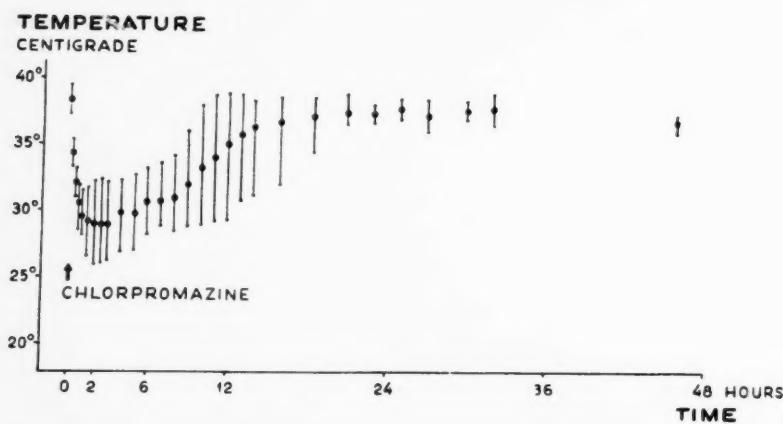


Fig. 3. — The hibernation effect and the range produced by $1/10$ LD₅₀ chlorpromazine (Largactil) on 30 adult Swiss Albino Webster male mice.

chlorpromazine used the mean temperature fell about 29 degrees centigrade in one hour. Shortly after this the temperature starts to rise again for about 3—4 hours. Twelve hours after the injection the mean temperature was 35°C but the range was then at its maximum (29—38°C). The body temperature of the whole group normalized after 21 hours and showed a normal range (2—3 degrees centigrade). The individual variation can be seen from Fig. 3. The range is very narrow, which shows that a uniform mice strain is very suitable for highly specific hibernation studies.

Regarding intra-individual variation, a test animal with an

excessively low body temperature after 2 hours' hibernation, for instance, could have an extremely high body temperature after 12—15 hours, and vice versa.

Weight and Mortality. — One month after the experiments the body weight had fallen a full 10%, and 20% of the 30 test animals had died.

DISCUSSION

In 1931 Buschke and Ollendauer (2) and in 1951 Nacke (8) observed the relationship of stress to body temperature by taking precision measurements of the body temperature of mice. Nacke pointed out that the temperature rise caused by the measuring manipulations was very disturbing whether performed quickly or not. He therefore rejected the use of the slow ordinary mercury thermometers. The intra- and inter-individual variations have not, however, been investigated.

In the voluminous literature on hibernation, these individual differences in body temperature have not been studied in a uniform mice-strain, the special purpose of this investigation.

The *diurnal rhythm* of the body temperature is comparatively even and highly dependent on the activity of the test animals.

The rise in body temperature following stress is surprisingly marked (from about 36—38°C to 39—40°C). Inter- and intra-individual variations notwithstanding, the trend is very distinct.

On *hibernation* induced by a chlorpromazine dose equal to approx. 1/10 of LD₅₀ the mean body temperature of this mouse strain sank almost 10 degrees centigrade in two hours. We noticed that the injection of chlorpromazine solution (or the equivalent amount of saline of the same temperature) often caused a rapid fall in rectal temperature of 1—2 degrees centigrade, and this in spite of the fact that the injection procedure might constitute a very considerable stress. This phenomenon must be interpreted as a consequence of the lower temperature (17—19°C) of the solutions. Buschke *et al.* (1931) also noticed this paradoxical temperature fall on giving subcutaneous injections (0.5—0.6 ml) of milk to mice (2). The range during hibernation was very wide, but the trend was quite distinct.

Making careful body temperature measurements, for instance by testing for pyrogens and antipyretic solutions (7), will reveal

how important it is to develop a reliable technique and how essential it is to have a highly sensitive precision thermometer. Repeated readings must be taken to obviate differences arising from the state of the animal's activity. To avoid activity variations (fighting, sexual activity etc.) it is recommended that the mice be housed separately, one per cage.

In hibernation especially, radiation and transfer of heat from the hands to the test animal and the applicators must be avoided as far as possible. The environmental temperature must be constant. This enables the chlorpromazine to transform the homotherm to poikilotherm to some extent (3).

P. Suomalainen observed that during the *natural* hypothermia (hibernation) of hedgehog (*Erinaceus europaeus*) in winter a fall in barometric pressure usually aroused the animal, whereas a rise sent it to sleep (and the body temperature fell) (9). This mammal is a relatively primitive creature with incompletely developed thermoregulation. It is tempting to assume that if chlorpromazine is able to transform the homothermal animal to a poikilothermal, then in *artificial* hibernation, too, changes in the body temperature should follow meteorological variations. The same dosage of chlorpromazine given to the same mice strain, however, resulted in barometric pressure — changed temperature differences which were very slight.

The use of animals less than 20 g in weight is not recommended since the body temperature of juvenile mice is very labile (8). The use of a genetically uniform strain of mice, bred in similar conditions and preferably of the same sex, is also desirable.

SUMMARY

The normal diurnal body temperature rhythm and the variations following stress, chlorpromazine hibernation and different states of activity were followed in an experimental study of 30 adult male mice of a uniform strain (Swiss Albino Webster). It was found that the diurnal rhythm was comparatively even. However the intra- and inter-individual body temperatures both varied relatively greatly, depending on the state of activity. Under intensive stress, such as fighting, the body temperature rose 3–4 degrees centigrade in a few minutes. During hibernation induced

with 30 mg of chlorpromazine per kg body weight, *i.e.*, c. 1/10 LD₅₀, injected subcutaneously, there was a fall in body temperature of 7—10 degrees centigrade. The temperature normalised after some 15 hours. In discussing the results recommendations are made concerning precision measurements of body temperature, with special emphasis on individual differences and the use of a both genotypical and phenotypical uniform test animal strain as far as possible.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY UNIVERSITY
OF HELSINKI

BODY TEMPERATURE STUDIES ON DIFFERENT MICE STRAINS

A GENETIC STUDY

by

ALDUR W. ERIKSSON and OLE WASZ-HÖCKERT

(Received for publication September 24, 1958)

In body temperature studies on a uniform mice strain (7) we observed that the intra- and inter-individual body temperatures both varied relatively greatly, depending on the state of activity. Clear inter-individual properties, however, could be seen. An animal reacting to stress with a high body temperature rise, for instance, had this tendency throughout.

The inter-individual differences prompted a study of the body temperature properties of different mice strains. Although artificial hibernation studies with mice are fairly numerous, nothing has been published on hypothermic studies of genetically different strains, an important point for our own further studies. Since our observations may help to explain the divergent views of different authors concerning stress properties, dosage of chlorpromazine (LD_{50}) and hypothermic tendency, and because they are of interest genetically, we decided to publish this report.

Aided by grants from the Signe and Ane Gyllenbergs Foundation and the Sigrid Jusélius Foundation.

MATERIAL

The following highly inbred mice strains were used in our experiments:

1. Swiss Albino Webster, SAW, 30 adult males, weight 20—30 g.
2. English Albino Wiele, EAW, 12 adult females, weight 20—28 g.
3. Danish Albino Götze, DAG, 12 adult males, weight 23—39 g.
4. Swedish Albino Rockneby, SAR, 12 adult males, weight 23—30 g.
5. German Albino BLH, BLH, 5 adult males, weight 22—27 g.
6. a. German Black C 57, C 57, 10 adult males, weight 23—35 g.
Non-infected.
- b. German Black C 57, (C 57 infect.), 10 adult males, weight 23—35 g. Infected.

The pre-experimental and phenotypical conditions were kept as similar as possible (7). Every animal was marked to facilitate observations of individual variations in body temperature, weight, standing in the cage, aggressiveness, sexual activity etc.

TECHNIQUE

The temperature and of these six different mice strains were measured under normal conditions, after stress and during artificial hibernation.

For the technique used in electrical precision measurements of the rectal (sigmoideum) body temperature and in the chlorpromazine hibernation we refer to our earlier experiments (7, 11). The animals were observed and stressed about a week before the experiments proper with the aim of discovering whether a correlation existed between the inter-individual variations to stress and in hibernation, *i.e.* the spread of the curves was studied.

The stress studies comprised rectal temperature measurements at 2½—4 minute intervals. On each occasion, the handling of the test animal produced in it a state of fright, strain and anxiety to which it reacted by kicking and biting and with a rise in body temperature.

Hibernation was induced as described in our earlier studies (7, 11). After repeated control of the normal body temperature, every test animal in order of number was given 30 mg/kg body

weight (about 1/10 of the mean lethal dose, LD₅₀, for SAW mice) of a sterile freshly-made 0.1% chlorpromazine hydrochloride solution (Largactil May & Baker).

RESULTS

Measurements repeated on different days and during different states of normal physiological activity elicited no divergences in mean temperature between the six different strains.

The effect of stress on the body temperature is seen in Fig. 1. The stress-releasing factors were exactly the same. This notwithstanding, there was a significant difference in the body temperature increase induced by the efforts of the test animals. With the SAW strain, the rectal temperature responded to minor (measurement) stress after 20 minutes with a rise of about 1½ degrees centigrade. After a major stress the rise was 3—4 degrees centigrade in quite a

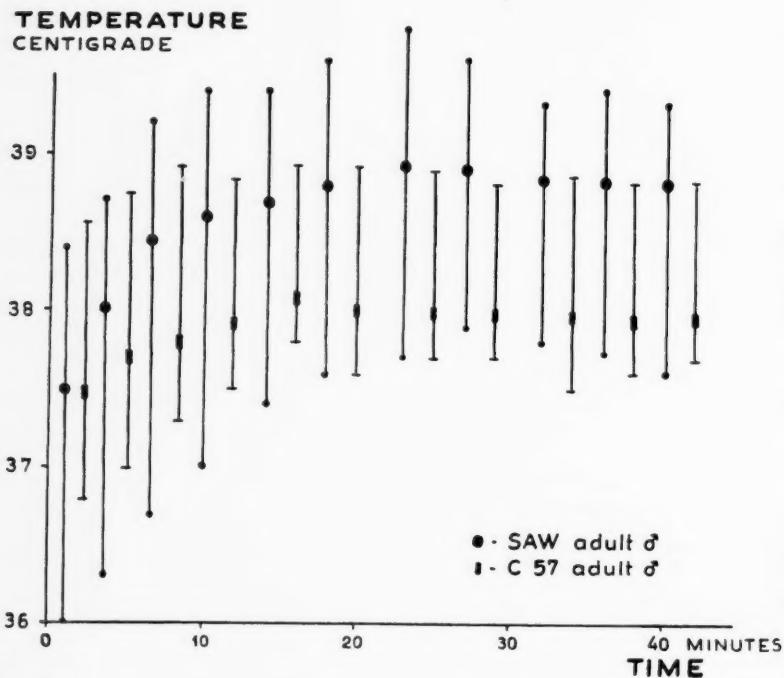


Fig. 1. — Body temperature and range following minor stress of 30 Swiss Albino Webster mice and 10 German Black C 57 mice.

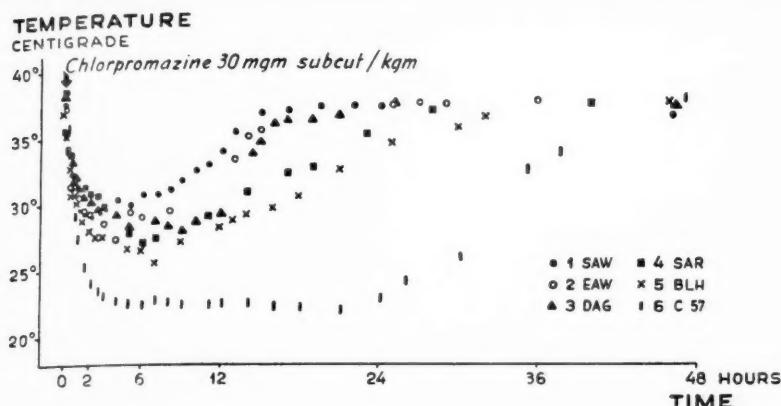


Fig. 2. — The chlorpromazine hypothermic reaction of six different mice strains.

short time. With the black C 57 strain the rectal temperature rose in response to an identical minor stress by only 0.5 degrees centigrade. Major stress, nevertheless, evoked a body temperature rise of as much as 2—3 degrees centigrade in individual black mice. The temperature range of the SAW group was wider (Fig. 2). A possible reason for this is that the non-infected black C 57 strain comprised only 10 test animals, $\frac{1}{3}$ of the SAW total.

Concerning the Largactil-induced hibernation, there was a divergence in the temperature curves between the different mice strains, as will be seen in Table 1 and Figs. 2 and 3. This body temperature difference was very distinct between the SAW and C 57 strains. The hibernation curve for the SAW strain reached its low within 2—3 hours, the curve for the black C 57 strain not until 21 hours had elapsed. Three hours after the chlorpromazine injection the mean body temperature of the SAW group was about 29°C, that of the C 57 strain as low as c. 23°C — a difference of 6 degrees. The C 57 low (22.2°C) was not reached until the SAW temperature had already normalised. At this stage there was consequently a body temperature difference of no less than 15 degrees centigrade. With the C 57 strain the body temperature normalised first after 48 hours, with the SAW within 18—21 hours. As the figures show, the other mice strains came somewhere between SAW and C 57.

Study of the inter-individual variations in the different strains showed that test animals which react to stress with a higher body

TABLE I
HYPOTHERMIC REACTION OF SIX DIFFERENT MICE STRAINS TO 30 MG OF CHLORPROMAZINE/KG GIVEN SUBCUTANEOUSLY

Strain	Number of Animals	Mean Body Temperature during Hibernation										Post-experimental Mortality			
		0	½	1	2	4	6	8	12	18	24	36	48 hrs.	4 Days	Mean Weight Loss
Controls SAW	30	36.6				37.0	37.3	37.2	37.1	37.0	37.0	36.8	36.6	37.0	
1. SAW	30	37.5	32.1	29.8	29.1	30.2	30.7	31.0	33.8	37.0	37.3	36.7	36.7	1.7 g	10 %
2. EAW	12	37.4	31.7	30.6	29.3	28.7	29.1	29.7	34.4	36.0	37.5	37.8	37.5	2.0 g	0 %
3. DAG	12	38.2	33.3	31.3	30.1	29.2	28.8	28.3	29.8	36.5	37.2	37.5	37.4	1.9 g	8 %
4. SAR	12	38.1	33.9	31.7	30.9	27.5	27.1	28.0	30.0	32.5	35.4	37.0	37.2	3.5 g	20 %
5. BLH	5	37.5	32.8	30.2	28.0	27.2	26.4	26.0	28.2	30.5	34.0	36.8	37.5	1.8 g	0 %
6. C 57	10	38.2	31.9	27.5	24.2	22.8	22.5	22.7	22.5	22.3	23.0	32.9	37.9	2.2 g	20 %
Infect. C 57														80 %	100 %

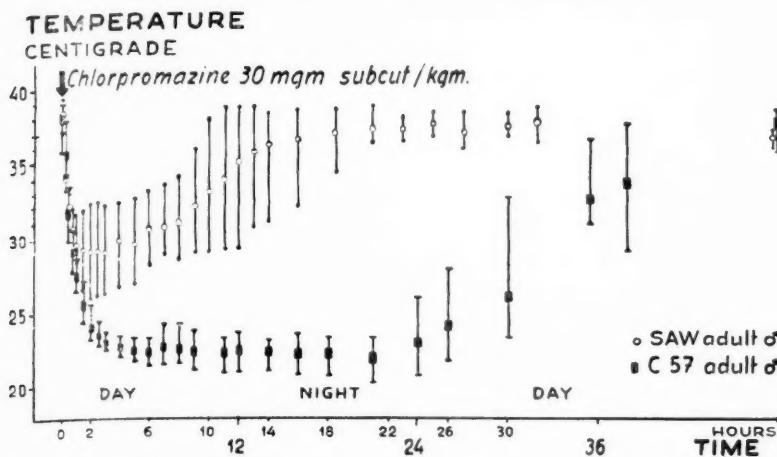


Fig. 3. — The mean body temperature fall and range, induced with the same dose of chlorpromazine, of two genetically different mice strains.

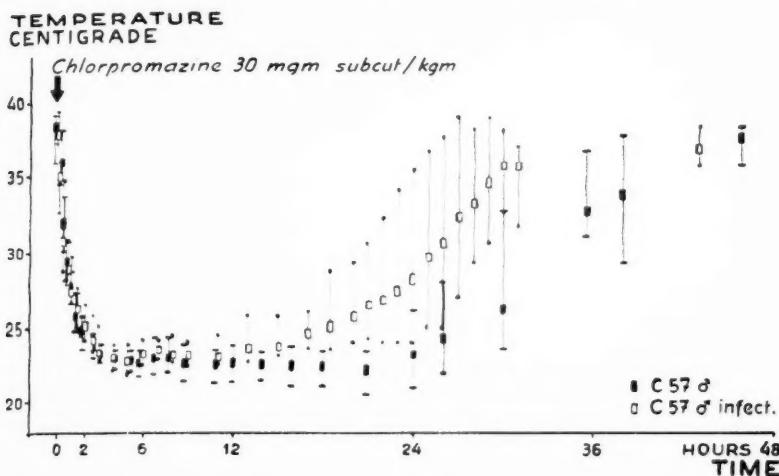


Fig. 4. — The mean body temperature fall and range following the same dose of chlorpromazine, of a non-infected and an infected group of the same mice strain.

temperature rise tolerate better the effect of the chlorpromazine drug. They have a lower hypothermic curve.

Four days after starting the hibernation experiments the greatest weight losses were recorded in groups 4, 5 and 6 — on an average 3.1, 1.8 and 2.2 g respectively.

The *post experimental mortality* was highest in strains 4 and 6. Two months after the start of the experiments all the animals in groups 4 and 6 had died.

From Table 1 and Fig. 4 it will be seen that black C 57 mice inoculated intraperitoneally with *Mycobacterium tuberculosis* H 37 Rv 4 months earlier showed a definitely smaller hypothermic tendency with chlorpromazine. Histological examination revealed clear tuberculous changes (tubercles). A similar phenomenon was noted with the infected SAW mice.

Four weeks before the hibernation experiments, SAW mice inoculated intraorbitally with staphylococci had a distinctly smaller tendency vis-à-vis the hypothermic depth. The mortality with 30–100% of LD₅₀ of Largactil was clearly smaller than with non-infected mice, as might have been expected.

DISCUSSION

Our experiments with the different mice strains were conducted in conditions as nearly identical as possible: the same atmospherical conditions as far as possible (5, 10), the same sterile freshly-made light-protected chlorpromazine solution, the same precision electrical measurement-apparatus, an identical milieu and the same well-balanced food pellets and water ad libitum. The experiments were made at room temperature (17–19°C). None of the test animals had earlier received chlorpromazine or other drugs; earlier studies (11) have shown that animals become tolerant to the effect of the chlorpromazine. With two exceptions, sufficient male mice were available in the different highly inbred strains — they were taken by random sampling. The exceptions were the English Albino Wiele strain, of which only females were available, and the BLH Albino strain, of which only a few males existed in the country. These two groups were added to the material, however, since the results were clear enough.

The loss of weight in the chlorpromazine series was striking although the test animals received at the beginning of the experiments a considerable subcutaneous injection, 0.6–1.2 cc, of saline, i.e. about 3% of the body weight — and despite fact that the metabolic activity is considerably reduced at a body temperature 8–15 degrees centigrade below the normal. The animals did, it is true,

refuse food in the hypothermic state, but at normal body temperature the weight loss in fasting mice in the same time was not as striking.

The post experimental mortality figures (Table 2) indicate a parallelism between hibernation depth and mortality. The lower the fall in body temperature (irreversible cell changes) the sooner and the more frequently the animals died.

Animals which behaved like »kings of the cage», and consequently must have been in good condition, had a low disposition to hibernate (7).

What was surprising was that the test animals with a chronic infection reacted to chlorpromazine hibernation with a clearly shorter hypothermic state. This lower hypothermic disposition in mice inoculated with tubercle bacilli and staphylococci remains a paradox.

Just where the divergence in the hypothermic mechanism lies between the different mice groups cannot be concluded from these experiments. Through the blocking of the gamma-motor system (9) the body loses one of the most important mechanisms for the production of heat by increased contraction of the skeletal muscles (8). But the muscular shivering in all the different mice strains was in no correlation to the remarkable reduction in body temperature. Of all the strains only the black C 57 mice were clearly slacker than the others in the hypothermic state.

Referring to the chlorpromazine absorption and excretion studies on animals by Berti & Cima (2, 3) and Dubost & Pascal (6), it may be possible that the cause of the prolonged hypothermic state of the C 57 strain is a hereditary insufficiency in the elimination of the chlorpromazine drug.

The characteristics of an organism are fundamentally the outcome of the interaction of heredity and environment, though in ordinary circumstances heredity plays the more important rôle (12). Hence it may be that genetical differences between the six mice strains are the most important cause of the body temperature divergences.

SUMMARY

The body temperature variations following stress, chlorpromazine hibernation and different states of activity were followed in an experimental study of 91 adult mice of six different strains.

During normal physiological conditions there were no observed divergences in body temperature between the six different strains.

Under stress there was a distinct difference in the body temperature rise between black and albino strains.

During hibernation induced by subcutaneous injection of 30 mg of chlorpromazine per kg body weight (c 1/10 of the LD₅₀ of Swiss Albino Webster mice), there was a definite difference between albino and black mice in the temperature fall (about 7 degrees centigrade) and in the hypothermic duration (over 24 hours).

Individual variations were also observed: «kings of the cage» reacting to stress with a high temperature rise had a low chlorpromazine hypothermic disposition.

It was noteworthy that the mice with chronic infection had a lower hibernatory tendency.

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FROM THE THIRD MEDICAL CLINIC, UNIVERSITY OF HELSINKI, AND FROM
THE CENTRAL LABORATORY, KIVELÄ HOSPITAL, HELSINKI

THEOPHYLLINE PLASMA LEVELS AFTER ORAL
ADMINISTRATION OF THEOPHYLLINE SODIUM
GLYCINATE

by

TIMO SEPPÄLÄ and AIMO KÄRKELÄ

(Received for publication September 29, 1958)

Theophylline and many of its derivatives have a low solubility in water. Oral administration of these compounds often causes gastrointestinal irritation. Therapeutic plasma levels cannot therefore always be attained. New theophylline derivatives, readily soluble in water, are now available. One of these compounds is theophylline sodium glycinate.

The purpose of this work was to study, whether therapeutic plasma levels of theophylline can be attained by oral administration of theophylline sodium glycinate.

METHODS

Thirteen hospital patients in good condition and without any gastrointestinal disease were studied. Every test subject received a single dose of 0.9 g. of theophylline sodium glycinate¹ (equivalent to 0.45 g. of theophylline). Heparinized samples of venous blood were taken immediately before and 1, 2, 3, and 5 hours after the administration of the trial dose. The test subjects were in a fasting state for twelve hours before and during the experiment.

¹ The preparation used was Teoglysin (Orion). According to the declaration of the manufacturer one tablet contains 0.45 g. of theophylline sodium glycinate, which is equivalent to 0.225 g. of theophylline.

Theophylline content of the plasma was estimated by the ultraviolet spectrophotometric method described by Schack and Waxler (1). The double extraction process used in this method recovers more than 95 per cent of the plasma theophylline. In the original method the results are calculated by using the molar extinction coefficient of theophylline which has been stated to be 10.200 at wave length 277 mu. In our estimations a standard sample prepared from pure crystalline theophylline was included in each series of estimations. The results were calculated by comparing absorption of plasma samples with that of the standard sample.

Caffeine and barbiturates, which are known to interfere in the ultraviolet spectrophotometric estimation of theophylline, are also extracted from the plasma by the method of Schack and Waxler. Administration of these substances and other drugs was therefore avoided immediately before and during the experiments. The plasma sample taken before the administration of the trial dose was used as a »plasma blank». Ultraviolet absorption of this plasma sample (extinction between 0.05 and 0.10) was subtracted from the absorption values of samples taken after the administration of the trial dose. Thus, the ultraviolet absorption obtained may be considered to be due to theophylline only.

RESULTS

Theophylline plasma levels of the 13 test subjects 1, 2, 3 and 5 hours after oral administration of 0.9 g. of theophylline sodium glycinate are shown in Table 1 and Fig. 1.

The maximum concentration was observed in most cases after 1—2 hours. The highest individual concentration, 2.500 mg. per 100 ml., was observed after 1 hour (Test subject 4). Individual variations were comparatively great. The maximum concentration was reached later in the cases in which it was low. In all cases the theophylline plasma level exceeded 1 mg. per 100 ml. which is considered to be the minimum theophylline plasma level for the relief of bronchospasm (3). In eight cases the theophylline plasma level after 5 hours was still above 1 mg. per 100 ml.

TABLE 1

THEOPHYLLINE PLASMA LEVELS (MG. PER 100 ML.) AFTER ORAL ADMINISTRATION OF 0.9 G. OF THEOPHYLLINE SODIUM GLYCINATE

Test Subject No.	Time (hrs.)			
	1 hr.	2 hrs.	3 hrs.	5 hrs.
1	1.980	1.637	1.445	1.078
2	2.132	2.276	2.342	2.066
3	2.396	2.302	2.139	1.868
4	2.500	2.488	2.474	2.027
5	2.201	2.000	1.946	1.679
6	0.543	0.920	1.097	0.870
7	1.910	1.764	1.468	1.247
8	2.051	1.964	1.822	1.614
9	1.430	1.163	1.107	0.963
10	1.381	1.270	1.167	0.888
11	1.488	1.458	1.350	1.231
12	1.044	1.103	0.970	0.856
13	0.935	1.340	1.256	0.891
Mean	1.692	1.668	1.583	1.329
Standard deviation	± 0.662	± 0.508	± 0.506	± 0.462

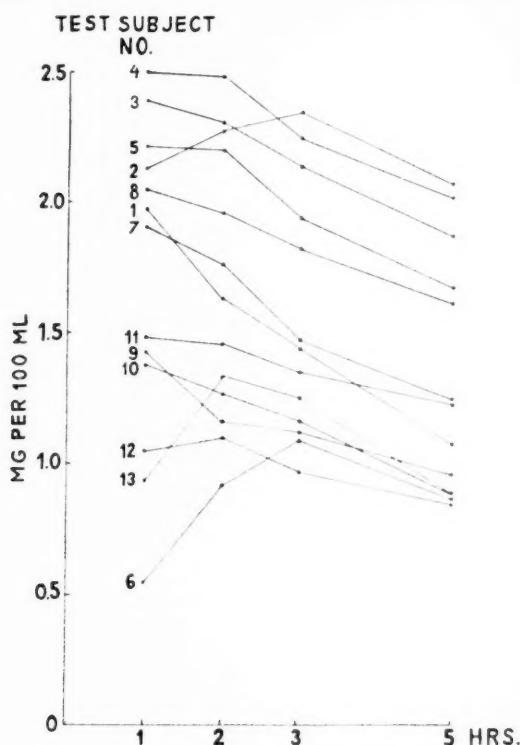


Fig. 1. — Theophylline plasma levels (mg. per 100 ml.) after oral administration of 0.9 g. of theophylline sodium glycinate.

DISCUSSION

The theophylline blood level after oral administration of theophylline sodium glycinate was first studied by Truitt *et al* (2). These writers administered daily four doses of 0.325 g. of theophylline sodium glycinate (equivalent to 0.16 g. of theophylline) and observed theophylline blood levels of 0.7—1.2 mg. per 100 ml. after 2—12 hours. Because the arrangement of experiments by these writers differs from that of ours, no direct comparison between their and our results can be made. Turner-Warwick (3) administered a single dose of 0.95 g. of theophylline sodium glycinate (equivalent to 0.47 g. of theophylline) and estimated the theophylline plasma level after 1, 2, 3, and 5 hours. In two of five cases the plasma level of theophylline exceeded 1 mg. per 100 ml. The highest value observed was 1.490 mg. per 100 ml.

It is worth noting that in our experiments the theophylline content of the plasma reached the therapeutic level, 1 mg. per 100 ml., in all the 13 cases after oral administration of a single dose of 0.9 g. of theophylline sodium glycinate. In eight cases the theophylline plasma level after 5 hours still was above 1 mg. per 100 ml. The trial dose did not cause gastrointestinal irritation or other side-effects in any of the test subjects. The therapeutic value of theophylline sodium glycinate, however, depends on the frequency of side-effects in maintenance treatment.

SUMMARY

Theophylline plasma levels were studied after the administration of a single oral dose of 0.9 g. of theophylline sodium glycinate (equivalent to 0.45 g. of theophylline). The therapeutic plasma level, above 1 mg. per 100 ml., was reached in all 13 test subjects, usually after 1—2 hours. In eight cases the theophylline plasma level after 5 hours still was above 1 mg. per 100 ml.

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EFFECT OF RESERPINE ON THE TOXICITY OF ADRENALINE AND NORADRENALINE AND ON THE PULMONARY EDEMA PRODUCED BY THESE SUBSTANCES

by

M. H. FRICK and PENTTI I. HALONEN

(Received for publication October 30, 1958)

It has been found that reserpine does not inhibit the pressor effect of adrenaline and noradrenaline in intact experimental animals but rather increases it (1, 14). Reserpine has also been found to increase the toxicity of adrenaline (11). This has been ascribed to weakening and inhibition of protective reflexes by reserpine. The cardiovascular effects of reserpine would therefore appear to be of a central nature. This is supported by the experiments of Harrison and Goth (8) with spinal cats, in which reserpine failed to increase the pressor effect of noradrenaline after spinal section. On the other hand, reserpine has been found to have a direct effect on the blood vessels. Tripod and Meier (15) were able to cause vasodilatation with reserpine in isolated rabbit coronary vessels, the tone of which had been raised with Pitressin or barium chloride. McQueen and Blackman (10) obtained a similar result. They also observed the long duration of the vasodilatative effect of reserpine. A feature identical in these two investigations was the raising of the blood vessel tone before reserpine was administered.

Schmilterlöw (13) demonstrated the occurrence of noradrenaline in extracts from blood vessels. A number of studies have shown that reserpine causes depletion of catecholamines from the organism and that such depletion also occurs from the blood vessel

walls (2, 3, 5, 7). Carlsson and ass. (6) observed that tyramine did not increase the blood pressure of cats treated with reserpine. This observation was confirmed by Burn and Rand (4), who also demonstrated that ephedrine and amphetamine have very little pressor action on reserpine-treated spinal cats. They concluded that sympathomimetic amines, such as tyramine, ephedrine and amphetamine, produce vasoconstriction by means of the noradrenaline in the blood vessel walls and that this effect is reduced or inhibited when the animal has been given reserpine.

The depletive effect of reserpine on catecholamines lasts for considerably longer time than its central sedative effect, which has been reported to weaken the protective reflexes and thus to increase the toxicity of adrenaline.

So far it has only been demonstrated that the administration of reserpine increases directly the toxicity of adrenaline. In this paper observations are reported of the effects of reserpine on the toxicity of adrenaline and noradrenaline and on the pulmonary edema produced by these substances. Special attention was paid on possible differences in effects of adrenaline and noradrenaline during and after central sedation caused by reserpine.

MATERIAL AND METHODS

The experiments were made with male white mice weighing 18—21 g. The preparations used were L-adrenaline (Adrenal Orion), L-noradrenaline (Noradrenal Orion) and reserpine (Serpasil Ciba), all of which were administered by intraperitoneal injection. The adrenaline is stated as base and the noradrenaline as bitartrate. The reserpine dose was 2 mg/kg in all the series. LD₅₀ was determined from a co-ordinate in which the dosage scale was logarithmic and the mortality ratio scale a probity one. The toxicity of noradrenaline was determined 3 and 72 hours after and that of adrenaline 72 hours after the injection of reserpine. Every animal was under observation for at least 2 hours after injection and the survival time was determined. After the injection 4—5 animals were kept in the same cage. The animals dying within 24 hours were included in the total mortality.

In studying the effect of reserpine on pulmonary edema doses of 5 mg/kg of adrenaline and 30 mg/kg of noradrenaline were used.

The mice were killed 15 minutes after injection, the thorax was opened immediately and the trachea ligated at the same level in all the animals. The lungs were exposed and the mediastinal fat, large blood vessels and heart were removed after ligation. The edemic fluid on the surface of the lungs was wiped off with paper. The lungs were weighed with an accuracy of 1 mg and the animals with an accuracy of 100 mg, and the lung/body ratio calculated by dividing the lung weight by the body weight and multiplying the quotient by 100. This ratio is designated as L/B ratio.

RESULTS AND DISCUSSION

The LD₅₀ of adrenaline was 5.36 mg/kg for control mice and 8.10 mg/kg when adrenaline was given 72 hours after reserpine. For noradrenaline the LD₅₀ was 42.6 mg/kg, 18.3 mg/kg 3 hours after reserpine, and 61.5 mg/kg 72 hours after reserpine. Thus 72 hours after reserpine injection the toxicity of both adrenaline ($t = 2.34$; $P = 0.02$) and noradrenaline ($t = 2.69$; $P = 0.01$) was lower than in the control series. Three hours after reserpine the toxicity of noradrenaline was greater than in the control series ($t = 4.45$; $P = 0.001$). The results are shown in Table 1.

TABLE 1

EFFECT OF RESERPINE ON THE TOXICITY OF ADRENALINE AND NORADRENALINE

Treatment	No. of Animals	Weight of Animals (g) \pm S.D.	LD ₅₀ (mg/kg) \pm S.E.
Adrenaline	53	19.6 \pm 1.9	5.36 \pm 0.38
Reserpine treatment + adrenaline after 72 hours	*		
Noradrenaline	50	19.4 \pm 1.7	42.6 \pm 5.05
Reserpine treatment + noradrenaline after 3 hours ..	30	20.9 \pm 1.4	18.3 \pm 2.71
Reserpine treatment + noradrenaline after 72 hours ..	44	17.7 \pm 2.4	61.5 \pm 4.18

When determining the toxicity of adrenaline and noradrenaline 72 hours after reserpine treatment it was also observed that the survival times were definitely longer than those of controls. This difference was especially clear after noradrenaline, as seen in Fig. 1, in which are shown the survival times up to 2 hours of the controls

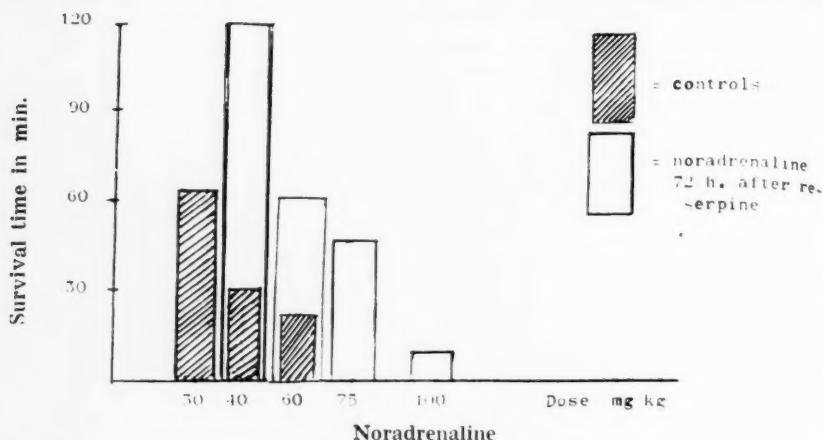


Fig. 1. — Effect of reserpine on survival time in noradrenaline-induced pulmonary edema.

The survival time is determined from deaths following within 2 hours after noradrenaline injection.

and the animals given various doses of noradrenaline 72 hours after the reserpine treatment. For adrenaline the observations were analogous, but both for controls and for reserpine-treated animals the survival times were shorter than after noradrenaline injection.

In studying the effect of reserpine on the severity of pulmonary edema, smaller doses of adrenaline and noradrenaline were used. A dose of 5 mg/kg of adrenaline or 30 mg/kg of noradrenaline produced in mice a pulmonary edema which the animals tolerated, on the average, for over 5 minutes. Reserpine administered 72 hours previously had no effect on the severity of adrenaline-induced pulmonary edema. The noradrenaline-induced pulmonary edema was more severe 3 hours after reserpine treatment than in the control animals ($t = 4.759$; $P = 0.001$) and 72 hours after reserpine less severe than in the controls ($t = 2.405$; $P = 0.02$). In determining the severity of pulmonary edema 72 hours after reserpine, the criterion used was the L/B ratio of controls 72 hours after reserpine. This ratio was 0.99, while the L/B ratio of intact controls was 0.76. The increased L/B ratio in the reserpine-treated mice was due in part to loss of body weight caused by reserpine and in part to an increase in the absolute weight of the lungs of

TABLE 2

EFFECT OF RESERPINE ON PULMONARY EDEMA PRODUCED BY ADRENALINE AND NORADRENALINE

Treatment	No of Animals	Weight of Animals (g) \pm S.D.	Lung/Body Ratio \pm S.E.
Controls	12	20.4 \pm 1.2	0.76 \pm 0.03
Reserpine controls after 72 hours	10	18.5 \pm 1.8	0.99 \pm 0.05
Adrenaline 5 mg/kg	10	17.6 \pm 1.0	1.19 \pm 0.08
Adrenaline 5 mg/kg 72 hours after reserpine	10	16.9 \pm 1.8	1.39 \pm 0.09
Noradrenaline 30 mg/kg	10	17.8 \pm 1.2	1.22 \pm 0.07
Noradrenaline 30 mg/kg 3 hours after reserpine	10	17.8 \pm 1.8	1.60 \pm 0.09
Noradrenaline 30 mg/kg 72 hours after reserpine	10	17.2 \pm 0.8	1.20 \pm 0.08

the reserpine-treated mice during 72 hours. The data on the severity of the pulmonary edema are shown in Table 2.

Milošević has demonstrated that the toxicity of the adrenaline in mice increases immediately after the injection of reserpine (11). The results obtained in the present study demonstrate that also the toxicity of noradrenaline increases during reserpine sedation and that the pulmonary edema developing during this time is more severe than in the control animals. On the other hand, 72 hours after the injection of reserpine, when the sedation had passed but there still was a depletion of catecholamines, the toxicity of both adrenaline and noradrenaline was lower than in the controls. At this time the pulmonary edema produced by noradrenaline was also milder than that of the controls, whereas no difference from the controls was seen in the adrenaline-induced pulmonary edema. The administration of reserpine as such appeared to increase the fluid content of the lungs, for 72 hours after the reserpine injection the weight of the lungs was greater in these animals than in the controls. In human subjects, reserpine treatment has been reported to cause pulmonary congestion and some cases of cardiac failure have been observed (9, 12). These have been ascribed to possible salt and water retention caused by reserpine. The cause of the increased toxicity of adrenaline and noradrenaline during sedation may possibly be a deficient function of protective reflexes. The

post-sedation decrease in adrenaline and noradrenaline toxicity may perhaps be associated with the catecholamine depletion produced by reserpine. It may be postulated that when adrenaline or noradrenaline is injected into an animal whose catecholamines have been depleted by reserpine, larger amounts may be necessary to produce the toxic effect than in intact animals, since a part of the injected catecholamines are consumed in supplying the depleted organism.

SUMMARY

Experiments were carried out with mice to determine the toxicity of adrenaline 72 hours after and of noradrenaline 3 and 72 hours after the injection of reserpine (2 mg/kg) and to observe the severity of pulmonary edema produced by these substances during the same periods. It was observed that the toxicity of both adrenaline and noradrenaline was lower 72 hours after reserpine treatment. The toxicity of noradrenaline 3 hours after reserpine was greater than in the controls and the noradrenaline-induced pulmonary edema was more severe. At 72 hours after reserpine, on the other hand, the pulmonary edema produced by noradrenaline was milder than that in the controls, whereas adrenaline-induced pulmonary edema did not differ from the edema in the control animals.

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FROM THE INSTITUTE OF OCCUPATIONAL HEALTH, HELSINKI

ASSAY OF GASTRIC SECRETORY INHIBITORS BY MEANS OF CARBAMINOYLCHOLINE

by

ESKO KARVINEN

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It has been demonstrated by Gray and Ivy (5) and Necheles *et al.* (11) that acetyl- β -methyl choline is a potent stimulator of canine gastric secretion. However, the response of total stomach pouches to repetitive subcutaneous administration declines with time and the compound shows a reversal effect with increasing dose. It was considered worth-while to adapt a cholinergic agent for quantitative determination of gastric secretory inhibitors in the same fashion as continuous histamine tests have been used by Code *et al.* (4) and by Karvinen and Karvonen (9). This type of assay would greatly aid in studying the mode of action of individual drugs on gastric secretion as well as the time course of the response. In the present study, successive fixed doses of carbaminoylcholine were utilized as recommended by Grossman for histamine stimulation (6). A fixed dose rather than a fixed response to the stimulating agent was sought (4, 6).

METHODS

Two healthy Heidenhain pouch dogs (7) were used. The pouches represented about 25% of the parietal mucosa. Absence of physiologically significant vagal innervation of the pouches was verified by injecting 2 units/kg body weight of insulin intravenously and

observing the resulting HCl output. This amounted to 0.05 and 0.02 milliequivalents of HCl per hour in the two dogs respectively. The response to the sight and smell of a fish and bone meal was negligible. The residual volume of the pouches was determined by instilling phenosulphonphthalein according to Öbrink (12). The residual volumes were found to be 4.5 and 6.2 cc. respectively.

Before each experiment the dogs were fasted overnight. During the experiment they were kept standing in a Pavlov Holder. Every ten minutes a subcutaneous injection of carbaminoylcholine chloride (May & Baker) in saline was given. In some experiments histamine dihydrochloride (Imido Roche) was administered similarly. Every 20 minutes all the gastric juice that flowed out of the pouch through a permanent rubber catheter was collected and its volume was measured.

The total acidity of the gastric juice was titrated with 0.1 N NaOH by using phenolphthalein as an indicator. The free acidity was titrated with dimethylaminoazobenzene as an indicator.

RESULTS

Maximum Response to Histamine. — Table 1 shows the maximum response of the Heidenhain pouches to subcutaneous histamine injected every ten minutes. The maximum response to histamine instead of carbaminoylcholine was determined because the maximum response to cholinergic agents is difficult to establish,

TABLE 1
MAXIMUM GASTRIC SECRETORY RESPONSE TO CONTINUOUS STIMULATION BY HISTAMINE

	Dose of Histamine γ/10 Min. Kg	Secretory Volume cc. Hour	Total Acid Millieq Liter	Acid Output Millieq Hour
Dog No. 1	8	4.8	154	0.7
	16	9.9	158	1.6
	32	16.5	158	2.6
	64	16.8	157	2.6
Dog No. 2	3	13.5	120	1.6
	6	15.9	134	2.1
	12	21.6	131	2.8
	24	17.9	133	2.4

the peak response being of short duration and subjected to dose reversal (5).

Continuous Response to Carbaminoylcholine. — The dose of carbaminoylcholine given every ten minutes was adjusted to produce approximately 25 per cent of the maximum secretory rate that was produced in response to histamine stimulation in the above experiments. The 25 per cent secretory rate (SR 25) was chosen because at this secretory level the inhibition of gastric secretion by various agents has been found to be more sensitive than when other secretory rates are used (4). The dose of carbaminoylcholine that was required to produce SR 25 was found to be $1.5\gamma/10\text{ minutes}/\text{kg}$ body weight in Dog No. 1 and $0.6\gamma/10\text{ minutes}/\text{kg}$ body weight in Dog No. 2. These doses were then used in all subsequent experiments. In Figure 1, the secretory response to repetitive carbaminoylcholine stimulation in 4 experiments with each dog is recorded. It may be noted that a fairly constant secretory response is produced by this method. First it takes about 90 minutes until the peak response to the repetitive stimulation is elicited. Then, a quite even plateau is reached and there is very little decline in secretory volume and acid output during the four hours of experiment. The HCl concentration of the gastric juice remains very stable.

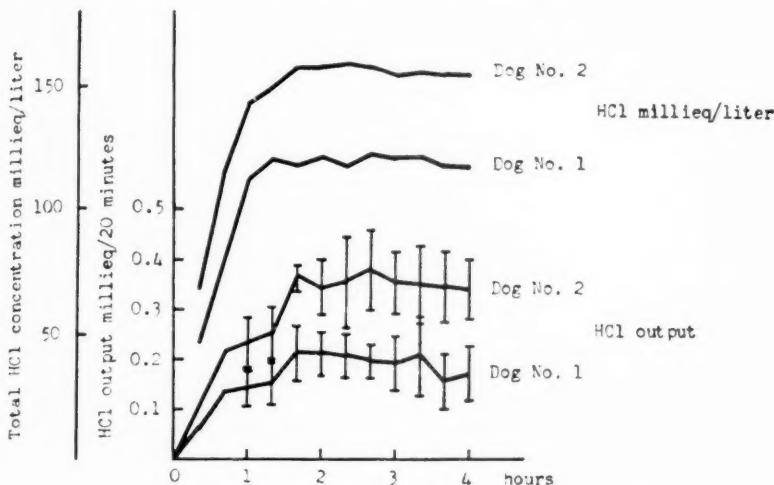


Fig. 1. — Gastric secretory response SR 25 of two Heidenhain pouch dogs to subcutaneous injections of carbaminoylcholine (Dog No. 1 $1.5\gamma/10\text{ min./kg}$ and Dog No. 2 $0.6\gamma/10\text{ min./kg}$). The mean values and standard deviations for 20 minute collections of gastric juice in 4 experiments with each dog.

In Table 2, the secretory response SR 25 during the second, third and fourth hour of repetitive carbaminoylcholine stimulation is shown. In spite of a slight decline during the fourth hour, the total acid output is quite stable having a coefficient of variation of 21 and 14 per cent in Dog No. 1 and 2 respectively.

It should be noted that SR 25 is not the maximum secretory rate in response to carbaminoylcholine stimulation. In Table 3, it is seen that large repetitive doses of carbaminoylcholine resulted in higher secretory rates at least for shorter periods of time.

Effect of a Secretory Inhibitor. — Insulin hypoglycemia is known to exert a potent inhibitory action on the secretion of HCl by denervated gastric pouches (2, 3, 9). In Fig. 2, the potency of insulin to inhibit the continuous gastric secretion induced by repetitive carbaminoylcholine stimulation is demonstrated. Figure 3 shows the effect of insulin hypoglycemia on the HCl concentration of the gastric juice.

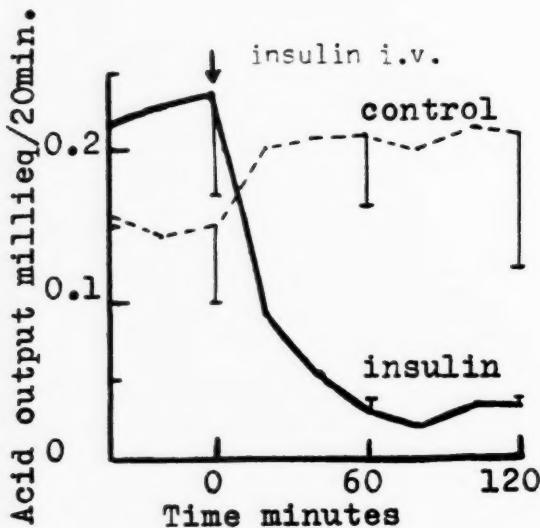


Fig. 2. — Effect of insulin (2 units/kg) on the carbaminoylcholine stimulated gastric secretion SR 25 of the Heidenhain pouch dog No. 1. Four experiments with insulin and four experiments with no insulin. Vertical bars show standard deviations.

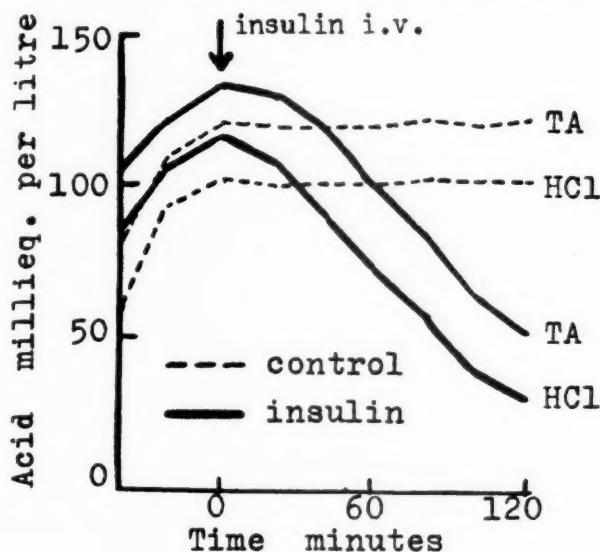


Fig. 3.—Effect of insulin (2 units/kg) on the total (TA) and the free (HCl) acidity of the carbaminoylcholine stimulated gastric secretion SR 25 of the Heidenhain pouch dog No. 1. Four experiments with insulin and four experiments with no insulin.

Comparison with a Continuous Secretory Response to Histamine.

The characteristics of a secretory response to small subcutaneous doses of histamine repeated every ten minutes are recorded in Table 4. The dose of histamine was initially adjusted to elicit SR 25 and the same dose was then used throughout. In Dog 2, the response to histamine is similar to the response to carbaminoylcholine. Dog 1, however, showed a higher response to the fixed dose of histamine ($8 \gamma/10$ minutes/kg body weight) than was its response to the $1.5 \gamma/10$ minutes/kg body weight of carbaminoylcholine.

In both dogs, the secretory response to histamine stimulation appeared to be more stable than the response to carbaminoylcholine. The coefficient of variation of the acid output was about 10% when histamine was used and 14 to 21% when carbaminoylcholine was used.

TABLE 2
GASTRIC SECRETORY RESPONSE SR 25 OF HEIDENHAIN POUCH DOGS TO REPEATED CARBAMINOYLCHOLINE INJECTIONS

Dog No.	Carbaminoyl-choline γ/10 Min./Kg	Number of Experiments	Hour of Experiment	Secretion Volume cc./Hour	Total Acid Millieq./Liter	Acid Output Millieq./Hour	Mean Acid Output Millieq./Hour 2nd through 4th Hour with Standard Deviation and Coeff. of Variation (%)
No. 1	1.5	4	2nd	4.9	120	0.6	0.58 ± 0.12 (21%)
			3rd	5.0	120	0.6	
			4th	4.5	119	0.5	
No. 2	0.6	4	2nd	6.4	151	0.9	0.99 ± 0.14 (14%)
			3rd	6.8	153	1.1	
			4th	6.6	155	1.0	

TABLE 3
GASTRIC SECRETORY RESPONSE TO LARGE DOSES OF CARBAMINOYLCHOLINE

Heidenhain Pouch Dog No.	Carbamino-ylocholine γ/10 Min./Kg	Number of Experiments	Hour of Experiment	Secretion Volume cc./Hour	Total Acid Millieq./Liter	Acid Output Millieq./Hour
No. 1	5.0	1	1st	9.8	155	1.5
			2nd	5.4	151	0.8
No. 2	2.5	1	1st	10.7	153	1.6
			2nd	6.9	153	1.1

TABLE 4
GASTRIC SECRETORY RESPONSE OF HEIDENHAIN POUCH DOGS TO REPEATED HISTAMINE INJECTIONS

	Histamine γ/10 Min./Kg	Hour of Experiment	Secretion Volume cc./Hour	Total Acid Millieq./Liter	Acid Output Millieq./Hour	Mean Acid Output Millieq./Hour 2nd through 4rd Hour with Standard Deviation and Coeff. of Variation (%)
Dog No. 1 4 experiments	8	2nd	7.2	139	1.0	1.1 ± 0.1 (9%)
		3rd	8.1	148	1.2	
		4th	8.4	149	1.2	
Dog No. 2 4 experiments	2	2nd	6.6	144	0.9	0.9 ± 0.1 (11%)
		3rd	6.3	142	0.9	
		4th	6.6	150	1.0	

DISCUSSION

In the present work, it was demonstrated that a fairly constant continuous secretory response to repetitive cholinergic stimulation can be elicited. However, this response may be less stable than the gastric secretory response to histamine. Also, the gastric secretion produced by carbaminoylcholine given by the present method may show a slight tendency to decline with time. This decline — if any — is much less pronounced than the decline of the secretory response to larger doses of choline esters reported previously (5).

The continuous secretory response to repetitive doses of carbaminoylcholine may be used for a quantitative assay of gastric secretory inhibitors as was clearly demonstrated in the case of insulin. The results of the present assay of insulin by means of carbaminoylcholine are very similar to data obtained by Karvinen and Karvonen (9) who assayed the inhibitory effect of insulin hypoglycemia on Heidenhain pouch secretion by using the continuous histamine method of Code *et al.* (4).

On a weight basis, carbaminoylcholine exceeds histamine in its potency to stimulate gastric secretion. In the experiments on Dog 2, in which the acid output in response to histamine and carbaminoylcholine were equal, carbaminoylcholine was three times as potent as histamine on a weight basis ($2.0 \gamma / 0.6 \gamma = 3$). However, it should be noted that histamine is metabolized quite rapidly (8, 10) whereas carbaminoylcholine is not attacked readily by acetylcholinesterase (1). Thus the potencies of the two compounds cannot be readily compared mol for mol.

SUMMARY

A fairly constant gastric secretory response to repeated doses of carbaminoylcholine was demonstrated at a secretory rate which was 25 per cent of the maximum secretory capacity of the Heidenhain pouches. This secretory response may be used for a quantitative assay of gastric secretory inhibitors.

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FROM THE WIHURI RESEARCH INSTITUTE, HELSINKI

THE MUCOPROTEIN CONTENT OF THE SERUM IN ANAEMIA

by

EERO HOKKANEN

(Received for publication November 11, 1958)

The proteins of serum rich in carbohydrates and their subfractions isolated by various methods have received abundant attention during the recent decades. One of these subfractions, the serum mucoprotein (referred to as M. P. in the following), has furnished the subject to numerous investigations since Winzler and his coworkers published (in 1948) their method of determination (20). As a result of these investigations the M. P. content was found to be increased in connection with trauma and with diseases causing inflammatory, proliferative or degenerative tissue changes; on the other hand, it has been noted to be decreased in association with parenchymal lesions of the liver, certain endocrine disturbances and nephrosis (5).

In haemorrhagic anaemia inflicted to experimental animals, the polysaccharide content of the serum has been previously investigated with controversial results (6). Most recently, Werner (1949) has found the glucosamine content of the serum to increase clearly, at the same time as the total protein content goes down (19). On the other hand, Weimer and his coworkers could not observe any changes in the seromucoid, glycoprotein or total protein content in connection with anaemia slowly produced in experimental animals by repeated bloodletting, despite distinct lowering of the haemoglobin content (18).

Previously also some single anaemia patients belonging to various groups of anaemia have been investigated as to the poly-

saccharide content (1, 6, 10, 13, 14) and M. P. content (5, 11) of their serum. However, the results obtained in the said investigations are contradictory to some extent, and it was therefore thought to be appropriate to investigate the M. P. content of the serum with patients suffering of various kinds of anaemia.

METHODS

Determination of the serum M.P. has been carried out according to a modification (16) of Winzler's method, a departure from this being made only in the respect that two consecutive filtrations have been performed upon the perchloric acid precipitation in order to obtain a filtrate of greater clarity. The procedure of double filtration reduces the obtained M.P. figures as compared to single filtration; the M.P. content of the serum of 45 healthy persons was thus found to be 76.4 ± 18.8 mg%. The methodic error, calculated from 85 M.P. double determinations, was found to be 9.74 mg%.

The total protein determinations of the serum have been performed by colorimetry, using biuret reagent according to the method presented by Goa (1953) (3). The methodic error, calculated from 16 double determinations, was 0.13%.

The haemoglobin determinations have been made according to Sahli's method, and the figures stated are corrected Sahli values.

MATERIAL

Experimental haemorrhagic anaemia was induced in 15 rabbits of body weights varying between 1750 and 4400 g. Altogether 124 haemoglobin (Hb.) determinations, 92 serum M.P. determinations and 23 total protein determinations were performed. From the blood samples obtained at the first instance of bloodletting the Hb., serum M.P. and total protein were determined and the figures obtained were then used as normal values. For the tests, the experimental animals were divided into groups of five rabbits each, as follows: *Group 1*: Acute haemorrhagic anaemia was induced in the animals of this group, drawing from each animal 55—60 ml blood by cardiac puncture; on each subsequent day 5—15 ml blood were obtained for the determinations by bloodletting from the ear.

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veins. By this procedure a decrease of Hb. to 31—54% was effected in 2 to 5 days, the Hb. value increasing again after this period. This test group contained the biggest rabbits of 2100 to 4400 g body weight. — *Group 2:* In this group, subacute haemorrhagic anaemia was produced by letting from the ear veins, at first, 25—40 ml blood daily during 3 to 4 days and subsequently 5—15 ml each day for the determinations. The animals in this group had body weights between 2300 and 3315 g. — *Group 3:* In this group blood was let from the ear veins of each rabbit in a quantity of 10—20 ml daily until Hb. had gone down to 23—26%, which took a time of 9 to 17 days. In this group also the total protein content of the serum was determined in addition to the M.P. and Hb. determinations. These rabbits had body weights ranging between 1750 and 2600 g.

The series of anaemia patients comprised altogether 53 different cases, *i.e.*, 36 women and 17 men, their age varying between 11 and 76 years. Altogether 155 serum M.P. determinations and 154 Hb. determinations were performed. The control series consists of 45 healthy persons representing various age groups and both sexes. The anaemia patient series is subdivided into four groups as follows: *Group 1:* 14 anaemia perniciosa diphyllobotrica (A.P.D.) patients, *i.e.*, 7 women and 7 men. 28 serum M.P. determinations and 27 Hb. determinations were made with this group. — *Group 2:* 11 anaemia perniciosa cryptogenetica (A.P.C.) patients, *i.e.*, 8 women and 3 men. M.P. and Hb. determinations were made 31 each. — *Group 3:* 14 anaemia hypochromica essentialis (A.H.E.) patients, *i.e.*, 12 women and 2 men. M.P. and Hb. determinations were made 31 each. — *Group 4:* 14 anaemia haemorrhagica (A.H.) patients, *i.e.*, 9 women and 5 men. M.P. and Hb. determinations were made 52 each.

RESULTS

With the available 15 rabbits the Hb. content of the blood was found to be $80.9 \pm 1.93\%$ at the commencement of the test series, their serum M.P. content ranging from 79 to 200 mg %, mean: $123.7 \pm 8.50\text{ mg\%}$, and the total protein content of their serum being $7.08 \pm 0.11\%$. The serum M.P. of the 45 healthy persons serving as a control series ranged from 38 to 118 mg %, mean: $76.4 \pm 2.80\text{ mg\%}$.

EXPERIMENTAL HAEMORRHAGIC ANAEMIA IN RABBITS

The means of the Hb. values, serum M.P. contents and total protein contents in the different test groups are stated in Table 1.

TABLE 1
THE EFFECT OF HAEMORRHAGIC ANAEMIA ON THE MUCOPROTEIN CONTENT OF
THE SERUM IN THE RABBIT

	Mucoprotein				Haemoglobin	
	Mean (mg %)	Standard Error (mg %)	t	P	Mean (%)	Standard Error (%)
Normal values	123.7	8.50			80.9	1.93
Acute haemorrhagic anaemia: Third day	161.0	12.96	2.39	<0.05	45.4	1.45
Fourth day*	189.5	8.45	5.43	<0.001	49.0	5.80
Subacute haemorrhagic anaemia: Third day	128.2	15.88	0.23	—	42.6	1.64
Fifth day	189.4	27.54	2.27	—	31.0	1.05
Slowly developing anaemia: Second day	147.2	8.38	1.93	—		
Seventh day	122.0	14.23	0.12	—	35.0	2.38
15th day	102.8	7.28	1.88	—	28.2	1.68

* Four rabbits only

1. Acute haemorrhagic anaemia: On the third day of experiment the M.P. content had increased to 161 ± 12.96 mg% and on the fourth day, with four rabbits, to 189.5 ± 8.45 mg%. The Hb. content was then $49.0 \pm 5.80\%$. After the fourth day, further increase of the M.P. content was noted with three rabbits, while the other two rabbits already showed a distinct decrease. The Hb. content attained its lowest values, 31—54%, on the second to fifth day of experiment, after which it began to increase.

2. Subacute haemorrhagic anaemia: On the third day of experiment the serum M.P. content was 128.2 ± 15.88 mg%, and on the fifth day, 189.4 ± 27.54 mg%, i.e., values showing no deviation from normal. The Hb. values were 42.6 ± 1.64 and $31.0 \pm 1.05\%$ on the corresponding days. In three cases, the M.P. content was followed also on the subsequent days when the Hb. values were already on the increase, and a decreasing trend of the M.P. content

was noted in these instances, too. The lowest Hb. values, 26—35%, were attained on the fourth to fifth day.

3. Slowly developing haemorrhagic anaemia: No distinct changes could be observed in the M.P. values; thus, the M.P. content on the second day of experiment was 147.2 ± 8.38 mg%, on the seventh day, 122.0 ± 14.23 mg%, and on the fifteenth day, 102.8 ± 7.28 mg%. The corresponding Hb. values were, $35.0 \pm 2.38\%$ on the seventh and $28.2 \pm 1.68\%$ on the fifteenth day. The lowest Hb. values, 23—26%, were attained after 9 to 17 days of bloodletting. In this group also the total protein content of the serum was determined, its initial value for the rabbits participating in this test being $7.08 \pm 0.11\%$. On the tenth day since the commencement of the test the total protein content had gone down to $6.18 \pm 0.10\%$.

ANAEMIA PATIENTS

The means of the Hb. values and serum M.P. contents found for the different groups of patients are stated in Table 2.

TABLE 2
THE MUCOPROTEIN CONTENT OF THE SERUM OF ANAEMIA PATIENTS ON ADMISSION,
BY ANAEMIA GROUP

Group*	Number of Cases	Mucoprotein				Haemoglobin		
		Range (mg %)	Mean (mg %)	Standard Error (mg %)	t	P	Mean (%)	Standard Error (%)
Normal	45	38—118	76.4	2.80				
A.P.D.	11	46—235	104.2	14.84	1.84	—	42.7	4.20
A.P.C.	11	48—134	87.9	8.58	1.26	—	45.7	4.19
A.H.E.	14	12—149	86.0	7.57	1.19	—	42.2	2.85
A.H.	14	42—160	94.9	8.19	2.14	<0.05	44.7	3.63

* For the abbreviations used, see p. 79.

1. Anaemia perniciosa diphyllobotrica: The group consists of 14 patients, with Hb. on admission averaging $42.7 \pm 4.20\%$. The M.P. content ranged on admission between 46 and 235 mg%, mean: 104.2 ± 14.84 mg%, which does not deviate from normal. In five cases the serum M.P. content was followed during treatment (altogether 19 M.P. determinations) during the time which was required

to restore normal Hb., but no significant change of the M.P. content could be demonstrated.

2. Anaemia perniciosa cryptogenetica: The mean Hb. of the 11 patients constituting this group was $45.77 \pm 4.19\%$ on admission, and the serum M.P. content ranged from 48 to 134 mg%, mean: $87.9 \pm 8.58\%$. The mean M.P. content does not deviate from normal. In this group the M.P. content of nine patients was followed during treatment (altogether 29 determinations) but no positive changes could be demonstrated in any stage of treatment.

3. Anaemia hypochromica essentialis: The mean Hb. on admission of the 14 patients was $42.2 \pm 2.85\%$ and their serum M.P. content ranged from 42 to 149 mg%, mean: $86.0 \pm 7.57\%$, which does not deviate from normal. With the exception of two cases, two or three blood samples were taken of each patient in different stages of the treatment but no significant change of the M.P. contents during treatment could be noted.

4. Anaemia haemorrhagica: The group consisted of 14 patients with an average of their Hb. contents at the commencement of investigation of $44.7 \pm 3.63\%$, their serum M.P. content ranging from 42 to 160 mg%, mean: $94.9 \pm 8.19\%$, which is slightly elevated, as compared to normal. The changes of the M.P. content during treatment were followed in 11 cases (altogether 49 M.P. determinations were made), the results being stated in Table 3. On an average seven days after commencement of the investiga-

TABLE 3
CHANGES OF THE SERUM MUCOPROTEIN CONTENT OF ANAEMIA HAEMORRHAGICA PATIENTS DURING TREATMENT

	Number of Cases	Mucoprotein				Haemoglobin		
		Range (mg %)	Mean (mg %)	Standard Error (mg %)	t	P	Mean (%)	Standard Error (%)
On commencement of investigation	14	42—160	94.9	8.19	2.14	< 0.05	44.7	3.63
Seven days after commencement of treatment..	10	90—175	122.9	8.44	5.23	< 0.001	55.4	1.97
On conclusion of treatment ..	11	41—161	101.3	12.93	1.88	—	82.5	4.71

tion, when the mean of the Hb. values was $55.4 \pm 1.97\%$, the M.P. content ranged from 90 to 175 mg%, mean: 122.9 ± 8.44 mg%, which is clearly in excess of normal. At the time of the last sampling, on conclusion of the treatment, the M.P. content, now 101.3 ± 12.93 mg%, did not deviate from normal any more. The Hb. mean was then $82.5 \pm 4.71\%$.

DISCUSSION

When anaemia is rapidly induced in rabbits by drawing large blood quantities (55—60 ml) at once, a distinct increase of the M.P. content of the serum can be noted. Letting a somewhat smaller blood quantity (25—40 ml) at a time an anaemia developing at a slightly slower rate can be produced and in this case, too, an increasing trend of the M.P. contents is observed. When small blood quantities (10—20 ml) at one time are let each day, a state of very strong anaemia is attained in about two weeks and also a distinct decrease of the total protein content of the serum but the M.P. contents are not changed.

These results are in agreement with previous investigations, according to which acutely and subacutely induced experimental haemorrhagic anaemia in rabbits causes a relative as well as absolute increase of the serum glucosamine content (19), while on the other hand, by drawing about 20% of the total blood quantity from rats and guinea-pigs by cardiac punctures, slight anaemia is produced but no changes in the total protein, glycoprotein or sero-mucoid contents are noted (18).

The results of the present work are in support of the frequently pronounced opinion that the protein components of the serum rich in carbohydrates would be regenerated more rapidly and the regeneration of the other protein components containing less carbohydrates would take place at a slower rate although, in anaemic states, before the normalization of the haemoglobin content (18, 19). It is appropriate, however, also to keep in mind the promoting effect of the stress state caused by massive bleedings upon the serum M.P. content and also upon its total polysaccharide content (2, 4, 5, 7, 8, 9, 12, 15, 18), no such sudden stress state occurring in slowly induced anaemia. The same conclusions were drawn by Weimer and his co-workers, who found that the increase in

the glycoprotein and seromucoid content of the serum of guinea-pigs was due to the stress state caused by repeated cardiac punctures rather more than to the haemorrhagic anaemia (17).

The investigation relating to the M.P. content performed with anaemia patients revealed that in the anaemia perniciosa diphyllobotrica, anaemia perniciosa cryptogenetica and anaemia hypochromica essentialis groups the M.P. content is not different from normal at any stage of treatment. In these states the anaemia develops slowly as a rule, and the results are thus in good agreement with the circumstance observed in experimental haemorrhagic anaemias produced slowly by letting small blood quantities at a time, namely, that the M.P. content then remains within normal limits. In the anaemia haemorrhagica group, on the other hand, in which the anaemic state had set in rapidly after massive haemorrhage, the initial serum M.P. content values were slightly higher than normal; after one week on an average the increase of the M.P. content was distinctly notable and at the conclusion of treatment, when Hb. was normal, no significant difference as compared to normal could be observed any more. The results obtained are thus in full correspondence with the increases of the serum M.P. content in acute haemorrhagic anaemia established in the experimental work.

SUMMARY

In the present work the mucoprotein content of the serum in experimental haemorrhagic anaemia has been investigated with 15 rabbits and a control series consisting of 45 healthy persons, and with 14 anaemia perniciosa diphyllobotrica patients, 11 anaemia perniciosa cryptogenetica patients, 14 anaemia hypochromica essentialis patients and 14 anaemia haemorrhagica patients.

In acute haemorrhagic anaemia the serum mucoprotein content was found to increase from the fourth day of experiment onwards in the rabbits. In subacute and slowly induced haemorrhagic anaemia no changes of the mucoprotein content were noted.

In the anaemia perniciosa diphyllobotrica, anaemia perniciosa cryptogenetica and anaemia hypochromica essentialis groups no changes of the mucoprotein content of the serum were observed. In the anaemia haemorrhagica group the mucoprotein content of

the serum was somewhat elevated on admission and clearly elevated after one week, decreasing again after rectification of the anaemic state.

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CLINICAL EVALUATION OF A SIMPLE PRECIPITATION TEST FOR SYSTEMIC LUPUS ERYTHEMATOSUS

by

TEPPO VARTIO

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In the J. A. M. A., March 22, 1958, Jones and Thompson (2) introduced a very simple precipitation test for systemic lupus erythematosus. According to these authorities, the test seemed to differentiate systemic lupus erythematosus from periarteritis nodosa, scleroderma, dermatomyositis, rheumatoid arthritis, and rheumatic fever with extremely few false-positive results. The result of the test could be modified to a certain degree, e.g., by therapy with adrenocortical steroids or corticotropin, or by a remission, but it appeared to parallel the progress of the disease.

Lee and Schultz (3), later, concluded the evidence, from the data of a series of 95 patients, that the test described defines a nonspecific imbalance among the serum globulins, and although it is frequently positive in patients with systemic lupus erythematosus, its specificity and sensitivity are not great enough to warrant its use as a routine diagnostic method in this disease. In a recently study of Dubois *et al.* (1) the authors report, according to a series of 258 patients with various disorders, that the test is completely nonspecific. It is of value only when positive, and then it indicates the presence of hyperglobulinemia. As a diagnostic procedure for systemic lupus erythematosus, it should be abandoned.

MATERIAL, METHOD, AND RESULTS

In order to control the clinical usefulness of the test described we have performed it in 98 patients with various disorders. The method of Jones and Thompson (2) was followed in detail. The test reagent is a 12% solution of p-toluenesulfonic acid in glacial acetic acid. The test consists in adding 0.1 ml. of serum or plasma to 2 ml. of the reagent.

The composition of the series and the results appear from Table 1.

TABLE 1
RESULTS OF L.E. TESTS IN 98 PATIENTS WITH VARIOUS DISEASES

Diagnosis	Cases No.	Specimens No.	Results			
			+3	+2	+1	0
Lupus erythematosus disseminatus (verified by LE cells and/or biopsy)	10	13	2	2	0	9
Suspicious cases of LED	12	16	0	4	1	11
Arthritis rheumatoïdes	12	13	0	1	0	12
Febris rheumatica	7	7	0	0	0	7
Infectio acuta	7	7	0	2	0	5
Eczema allergicum	6	6	0	0	0	6
Carcinoma pulm. c. metast.	3	4	0	2	0	2
Carcinoma pancreatis c. metast. hepatitis	5	5	0	0	0	5
Pleuritis exsudativa	3	3	0	1	0	2
Pyelocystitis acuta	1	1	0	1	0	0
Miscellaneous diseases	32	32	0	0	0	32
Total	98	107	2	13	1	91

In 10 patients with LED the test was positive in three cases. In one untreated case the test was first negative and after two weeks positive. In another case treated during a half year with cortisone, ACTH and chloroquine, the test was first negative and after a month positive. An attempt was made to correlate the tests with sedimentation rate, CRP, and LE cells. In the LE test positive cases the sedimentation rate varied 20—125 mm/hour, the CRP was positive in all cases (from + 2 to + 4), and the LE cells negative in all cases. As seen from Table, the test is not specific for systemic lupus erythematosus.

SUMMARY

Our experience with a simple precipitation test for systemic lupus erythematosus, introduced by Jones and Thompson, agrees with the results of earlier investigations, that the test is not specific for this disease.

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FROM THE DEPARTMENT OF MEDICAL CHEMISTRY, UNIVERSITY OF HELSINKI

DEACETYLATION OF *P*-ACETYLAMINOBENZOIC ACID *IN VITRO* BY VARIOUS RAT TISSUE PREPARATIONS

by

MARTTI KOIVUSALO and TAPANI LUUKKAINEN

(Received for publication November 12, 1958)

Aromatic amines like sulphonamides and *p*-aminobenzoic acid are known to be readily acetylated in the animal organism *in vivo*. The acetyl conjugates formed are generally assumed to be excreted without decomposition from the organism (3, 5, 10—13). According to Krebs, Sykes and Bartley (8) the acetylation is irreversible in man and rabbit but can be reversed in the pigeon, dog and cat. Bettschart, Kohn and Bovet (1) have reported that perfusion of rat liver with a Tyrode solution containing monoiodoacetate and acetylsulphanilamide resulted in a partial deacetylation, which however, was not demonstrable when blood was used as perfusion fluid.

The poor acetylation of aromatic amines in various tissue preparations *in vitro* has been ascribed to an activation of the deacetylating enzyme system (1, 4, 8). Kohl and Flynn (6) have first shown in rat liver brei the presence of a deacylating enzyme system which hydrolyses N⁴-acyl-substituted sulphanilamide. Krebs *et al.* (8) have observed the hydrolysis of acetylsulphonamides by various tissue suspensions of the sheep. They have also suggested that the observed discrepancies between the acetylating power of sliced or minced tissues and the intact body in rat, guinea pig and rabbit may be connected with changes in the relative activities of the acetylating and deacetylating enzymes, brought about by slicing and mincing.

In studies in the acetylation of *p*-aminobenzoic acid in various rat tissue preparations (7, 9) in this laboratory it has been observed that although acetylation occurred in liver slices and cuttings, no activity was found in homogenates prepared from the same liver. One explanation possible for this difference would be an increased deacetylation activity in the homogenates. In the present paper results are presented from experiments in the deacetylation of *p*-acetylaminobenzoic acid in rat liver and kidney preparations.

METHODS

The experimental animals used were male and female rats of Wistar strain. The rats were killed by decapitation, their livers and kidneys were removed and placed on ice until used.

The *p*-acetylaminobenzoic acid was purchased from The British Drug Houses, Ltd., and the *p*-aminobenzoic acid from Schering-Kahlbaum A. G.

The composition of the incubation medium was as follows:

0.154 M sodium chloride, 200 ml

0.154 M potassium chloride, 8 ml

1.0 M sodium acetate, 0 or 8 ml

0.01 M *p*-acetylaminobenzoic acid solution in 0.01 M KHCO_3 , 8 ml

0.1 M potassium sodium phosphate, buffer pH 7.4, to make 400 ml

In the acetylation experiments 4 ml of 0.02 M neutralised *p*-aminobenzoic acid solution was substituted for the *p*-acetylaminobenzoic acid solution.

Both homogenates and small tissue cuttings were used as the enzyme source. Weighed amounts of liver tissue were homogenized in the aforementioned medium in a Büchner homogeniser and diluted with the same medium to make a 20 per cent homogenate. In each experiment 10 ml of this homogenate were used.

The tissue cuttings preparation was made as follows: 2 g of liver or 1 g of kidney was cut with scissors under 10 ml of the incubation medium into minute even sections.

The incubation flasks were gassed for 10 minutes with oxygen. Thereafter they were incubated in a temperature-controlled water bath at 37° for 4 hours with constant shaking. Duplicate samples of 1 ml were taken from each flask before and after incubation and transferred into centrifuge tubes containing 4 ml of 5 per cent trichloroacetic acid for deproteinization. The clear supernatant obtained after centrifugation was used for the analyses.

The method of Bratton and Marshall (2) was used for the determination of diazotizable amines. The determinations were made before and after hydrolysis with hydrochloric acid. The difference between the two was taken as the amount of acetylated conjugates.

RESULTS AND DISCUSSION

Results of typical series of experiments are presented in Tables 1 and 2.

No deacetylation was found to occur in any of the tissue preparations tested in mediums with or without added acetate. Liver

TABLE 1

DEACETYLATION OF *p*-ACETYLAMINOBENZOIC ACID (ACETYL-PAB) AND ACETYLATION OF *p*-AMINOBENZOIC ACID (PAB) BY VARIOUS RAT TISSUE PREPARATIONS IN A MEDIUM CONTAINING ACETATE

Tissue Preparation	Substrate	Amount of Tissue, g	Acetyl-PAB Found, μ moles	
			0 Hours	4 Hours
Liver cuttings	Acetyl-PAB	2	1.87	1.92
" "	"	2	1.92	1.93
" "	"	2	1.90	1.89
Liver homogenate	Acetyl-PAB	2	1.86	1.86
" "	"	2	1.88	1.87
" "	"	2	1.89	1.91
Kidney cuttings	Acetyl-PAB	1	1.86	1.89
" "	"	1	1.90	1.93
Liver cuttings	PAB	2	0	1.21
" "	"	2	0	1.18
Liver homogenate	PAB	2	0	0
" "	"	2	0	0
Kidney cuttings	PAB	1	0	0.37
" "	"	1	0	0.23

Total volume in incubation flasks 10 ml. Incubated for 4 hours at 37°. Gas phase oxygen. Amount of substrate 2 μ moles. The medium contained 200 μ moles of acetate per 10 ml.

TABLE 2

DEACETYLATION OF *p*-ACETYLAMINOBENZOIC ACID (ACETYL-PAB) BY RAT LIVER CUTTINGS IN A MEDIUM WITHOUT ADDED ACETATE

Tissue Preparation	Substrate	Amount of Tissue, g	Acetyl-PAB Found, μ moles	
			4 Hours	0 Hours
Liver cuttings	Acetyl-PAB	2	1.86	1.88
" "	"	2	1.87	1.90
" "	"	2	1.86	1.89
" "	"	2	1.86	1.89

Experimental conditions as in Table 1.

and kidney cuttings converted constantly *p*-aminobenzoic acid to acetyl derivatives, whereas no acetylation was found in liver homogenates. This inactivity of homogenates has been observed also earlier (7, 9). Because no deacetylation was demonstrated in homogenates it is apparent that the absence of acetylation in homogenates is not due to an activation of the deacetylating enzyme system, as has been suggested earlier (1, 4, 8).

SUMMARY

Deacetylation of *p*-acetylaminobenzoic acid has been studied in rat liver homogenates and cuttings and in rat kidney cuttings in mediums with and without added acetate. No deacetylation has been found to occur in any of the tissue preparations studied.

The liver and kidney cuttings converted considerable amounts of *p*-aminobenzoic acid to acetylated derivatives but no acetylation was observed in the liver homogenates.

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FROM THE DEPARTMENT OF MEDICAL CHEMISTRY, UNIVERSITY OF HELSINKI

URINARY METABOLITES OF *P*-AMINOBENZOIC ACID IN MAN

EFFECT OF BENZOATE

by

MARTTI KOIVUSALO, TAPANI LUUKKAINEN, TATU MIETTINEN,
and JAAKKO PISPA

(Received for publication November 18, 1958)

According to the investigations of Tabor, Freeman, Bailey and Smith (5), the principal urinary metabolites of *p*-aminobenzoic acid in man were *p*-aminohippurate, acetyl-*p*-aminobenzoate, acetyl-*p*-aminobenzoyl glucuronide and *p*-aminobenzoyl glucuronide. Only small amounts of free *p*-aminobenzoic acid and acetyl-*p*-aminohippurate were excreted after the administration of *p*-aminobenzoic acid. These same conjugates have been found also in rat urine (4) but there the acetyl-*p*-aminohippurate formed a large fraction of the total metabolites excreted. Administration of benzoate caused a marked change in the excretion pattern of *p*-aminobenzoic acid conjugates in the rat (4). After benzoate the only metabolite of *p*-aminobenzoic acid excreted in noteworthy amounts was acetyl-*p*-aminobenzoate.

In the present report it is demonstrated that appreciable amounts of acetyl-*p*-aminohippurate can be excreted also in man after administration of *p*-aminobenzoic acid. Simultaneous administration of benzoate inhibited totally the glycine conjugation of *p*-aminobenzoic acid, and the principal metabolites found in the urine after the administration of benzoate were acetyl-*p*-aminobenzoate and acetyl-*p*-aminobenzoyl glucuronide.

MATERIAL AND METHODS

The experimental subjects were 3 healthy 23--29 year old human males.

The dose of *p*-aminobenzoic acid was 1 g and it was taken per os with 400 ml of water. Urine was collected during a period of 4 hours after the administration of *p*-aminobenzoic acid. One week later the same dose of *p*-aminobenzoic acid together with 10 g of sodium benzoate was taken by the same persons and the urines collected as above.

The different metabolites of *p*-aminobenzoate were separated using the counter-current distribution technique of Craig (2). The solvent system was n-amyl alcohol and 0.1 M acetate buffer pH 3.4. 24 transfers were made in an all-glass apparatus.

The glucuronides were determined in the aqueous phase by the carbazol method of Dische (3). Before analysis the samples were extracted three times with ether to remove the n-amyl alcohol present.

The diazotisable amines were determined by the method of Bratton and Marshall (1) before and after acid hydrolysis. The determinations were made from both phases. *p*-Aminobenzoic acid and its metabolites were extracted from the organic phase with 0.1 N NaOH.

The distribution coefficients of *p*-aminobenzoic acid, *p*-aminohippuric acid and acetyl-*p*-aminobenzoic acid were determined as described earlier (4).

RESULTS

The results are presented in Tables 1—3 and in Figures 1—2.

The amounts of the different derivatives of *p*-aminobenzoic acid excreted in the urine during 4 hours after the administration of 1 g (7300 μ moles) of *p*-aminobenzoic acid are presented in Table 1. The largest fraction was *p*-aminohippurate amounting to 51 per cent of the total amount of *p*-aminobenzoic acid derivatives excreted. This value incorporates a small amount of *p*-aminobenzoyl glucuronide, which is not well differentiated from *p*-aminohippurate in the distribution system used. This is also seen from Fig. 1, in which one experimental run is presented. The next fractions in

TABLE 1
EXCRETION OF URINARY METABOLITES OF P-AMINOBENZOIC ACID (PAB)

	Total PAB Derivatives μ moles	PAB μ moles	PAH (+PABG) μ moles	Acetyl-PAB μ moles	Acetyl-PAH μ moles	Acetyl-PABG μ moles	Glucuronide μ moles
Subject 1 ..	5630	90	2765	405	1270	1100	1670
* 2 ..	6400	102	2945	736	1640	977	1660
* 3 ..	5830	99	3350	338	1376	667	1270
Mean values	5953	97	3020	493	1429	915	1533
% of total excretion ..		1.6	50.8	8.3	24.0	15.4	

Dose of PAB 1 g (7300 μ moles). Urines collected during 4 hours after administration of PAB. PAH = *p*-aminohippurate, these values incorporate also a small amount of *p*-aminobenzoyl glucuronide (PABG).

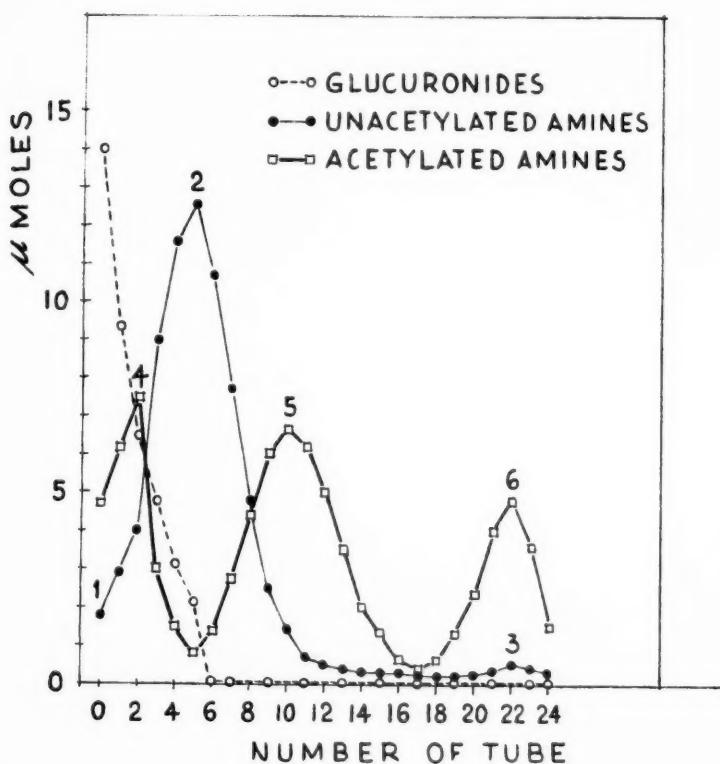


Fig. 1. — Excretion pattern of *p*-aminobenzoic acid and its conjugates in human urine.

Subject 2. 1. *p*-Aminobenzoyl glucuronide. 2. *p*-Aminohippurate. 3. *p*-Aminobenzoate. 4. Acetyl-*p*-aminobenzoyl glucuronide. 5. Acetyl-*p*-aminohippurate. 6. Acetyl *p*-aminobenzoate.

amount were acetyl-*p*-aminohippurate 24 per cent, acetyl-*p*-amino-benzoyl glucuronide 15 per cent and acetyl-*p*-aminobenzoic acid 8 per cent. Only 1.6 per cent of the total derivatives were excreted unconjugated. The value for the total glucuronides presented in the last column of Table 1 includes also other glucuronides than *p*-aminobenzoic acid conjugates.

The results of experiments in which 10 g of sodium benzoate were administered simultaneously with the 1 g of *p*-aminobenzoic acid are presented in Table 2 and in Fig. 2. It is seen that the administration of benzoate abolished totally the excretion of glycine conjugates of *p*-aminobenzoic acid. The largest fraction was now acetyl-*p*-aminobenzoic acid, which was 48 per cent. The absolute amount of acetyl-*p*-aminobenzoic acid excreted after benzoate was three times as large as without benzoate administration. The acetyl-*p*-amino-benzoyl glucuronide fraction was 39 per cent and the *p*-aminobenzoyl glucuronide fraction 10 per cent. The excretion of unconjugated *p*-aminobenzoic acid was 4 per cent, in μ moles this was nearly the same as without benzoate. The total glucuronide excretion increased very markedly, presumably owing to the formation of benzoyl glucuronides.

TABLE 2
EXCRETION OF URINARY METABOLITES OF *P*-AMINOBENZOIC ACID (PAB) AFTER
ADMINISTRATION OF *P*-AMINOBENZOIC ACID AND BENZOATE

	Total PAB Deriva- tives μ moles	PAB μ moles	PAH μ moles	PABG μ moles	Acetyl- PAB μ moles	Acetyl- PAH μ moles	Acetyl- PABG μ moles	Glucu- ronide μ moles
Subject 1	2413	148	—	240	1155	—	860	518
2	3930	137	—	236	1870	—	1687	707
3	3198	70	—	465	1510	—	1153	732
Mean values ..	3177	118	—	314	1512	—	1230	652
% of total excretion		3.7	—	9.9	47.7	—	38.8	

Dose of PAB 1 g (7300 μ moles), dose of sodium benzoate 10 g. Abbreviations as in Table 1.

Urines collected during a period of 4 hours after the administration of PAB and benzoate.

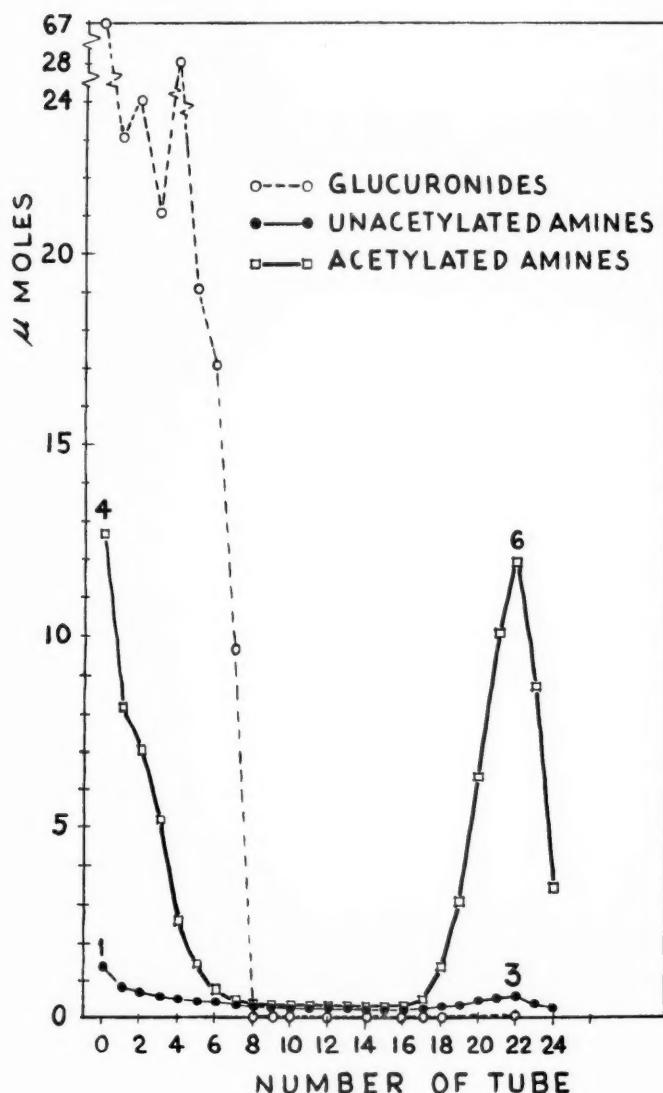


Fig. 2. — Excretion pattern of *p*-aminobenzoic acid and its conjugates after simultaneous administration of benzoate.
Subject 2. The numbers of the peaks as in Fig. 1.

TABLE 3

EFFECT OF BENZOATE ON THE EXCRETION OF ACETYLATED AND UNACETYLATED DERIVATIVES OF *P*-AMINOBENZOIC ACID

	Total Amount of PAB Derivatives Excreted μ moles	% of Dose Excreted	Acetylated Derivatives μ moles	Unacetylated Derivatives μ moles	Acetylation Percentage
PAB	5953	81.6	2837	3117	47.5
PAB + benzoate	3177	43.6	2742	432	86.6

Mean values from Tables 1—2.

During 4 hours 82 per cent of the administered *p*-aminobenzoic acid was excreted in the urine principally in the form of different conjugates. When benzoate was administered simultaneously the excretion fell to 44 per cent (Table 3). The excretion of acetylated conjugates was the same with and without benzoate. The excretion of unacetylated conjugates decreased about 86 per cent after the administration of benzoate, and thus the acetylation percentage increased from 48 to 87 per cent after the administration of benzoate (Table 3).

DISCUSSION

The urinary excretion pattern of *p*-aminobenzoic acid derivatives was very uniform in all the three test subjects examined in the present study. When *p*-aminobenzoic acid was administered to the test subjects, about 70 per cent of the total excretion consisted of glycine conjugates of *p*-aminobenzoic acid. One third of these was acetylated *p*-aminohippurate. Of the glucuronide conjugates the greater part was acetylated *p*-aminobenzoyl glucuronide. Tabor *et al.* (5) found only traces of acetyl-*p*-aminohippurate in human urine after administration of *p*-aminobenzoic acid, and the amount of acetylated glucuronide of *p*-aminobenzoic acid was smaller than that of *p*-aminobenzoyl glucuronide. The different doses of *p*-aminobenzoic acid used may explain the dissimilarity between the present results and those of Tabor *et al.* (5) observed in the excretion of glucuronide conjugates. The differences in the acetyl-*p*-aminohippurate excretion were probably not due to the different dosage. The relative amounts of *p*-aminohippurate, acetyl-*p*-amino-ben-

zoate and *p*-aminobenzoate were of the same magnitude in the present experiments as in those of Tabor *et al.* (5).

No glycine conjugates of *p*-aminobenzoic acid were found in the urine after the administration of *p*-aminobenzoic acid and benzoate. This was most probably due to competition between benzoate and *p*-aminobenzoic acid in the system forming hippuric acids. The total amount of *p*-aminobenzoic acid derivatives excreted decreased considerably after the administration of benzoate. The amount of acetylated derivatives was not affected by benzoate and the increase of the acetylation percentage observed after benzoate was thus not due to an increased acetylation.

SUMMARY

The urinary excretion of the different metabolites of *p*-aminobenzoic acid in man has been determined after the administration of *p*-aminobenzoic acid or *p*-aminobenzoic acid and benzoate. The metabolites were separated using counter-current distribution technique.

The principal metabolite excreted after the administration of *p*-aminobenzoic acid was *p*-aminohippurate. Next in order were acetyl-*p*-aminohippurate, acetyl-*p*-aminobenzoyl glucuronide, acetyl-*p*-aminobenzoic acid and *p*-aminobenzoic acid.

Administration of benzoate with the *p*-aminobenzoic acid abolished totally the excretion of the glycine conjugates of *p*-aminobenzoic acid. The principal metabolites found in the urine after the administration of benzoate were acetyl-*p*-aminobenzoate and acetyl-*p*-aminobenzoyl glucuronide.

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FROM THE RHEUMATISM FOUNDATION HOSPITAL, HEINOLA, FINLAND

THE BENTONITE FLOCCULATION TEST (BFT) IN RHEUMATOID ARTHRITIS

by

V. A. I. LAINE and H. JULKUNEN

(Received for publication November 26, 1958)

According to Bozicevich, Bunim, Freund and Ward (1) the BFT was positive in 78% of cases in a series of 41 rheumatoid arthritis patients.

In order to obtain more information about the clinical correlations of this reaction we have tested a larger group of verified rheumatoid arthritis cases.

MATERIAL AND METHODS

The series consisted of 100 adult cases (86 females and 14 males) of definite rheumatoid arthritis. These cases were selected from the clinical series of the Rheumatism Foundation Hospital, using the criteria of the A.R.A. Committee (Ropes, Bennett, Cobb, Jacox and JESSAR). Only cases with five positive criteria were taken into account. In every case the disease was active.

The BFT was performed on each case at least once, using the technique described by Bozicevitch *et al* (1). After a preliminary experimental investigation five sera were cross-checked in our laboratory and in the laboratory of the National Institute of Health, Bethesda, Maryland. The results from these two laboratories are in good agreement with each other.

Microscopic photographs of negative and positive tests are shown in Figs. 1 and 2.

The Waaler-Rose test was performed in 43 cases, using the method described by Rose *et al.* (3).

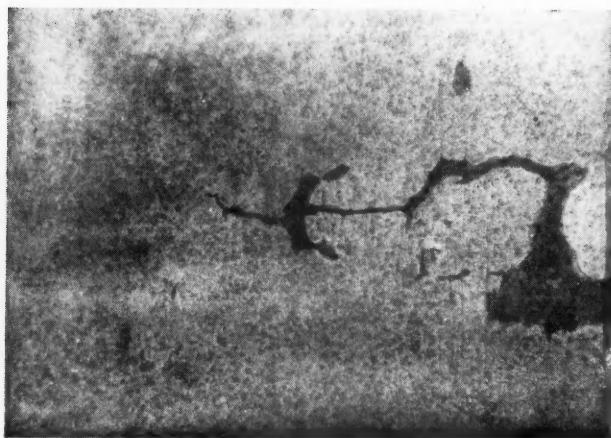


Fig. 1. — Negative BFT ($\times 375$)

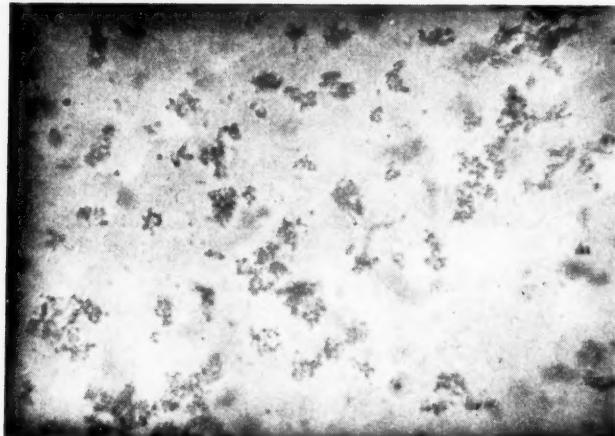


Fig. 2. — Positive BFT ($\times 375$)

RESULTS

64% of the cases showed a positive BFT. There was no correlation to be seen between sex incidence and age. The various stages (I-IV) were represented in equal proportion in the positive and in the negative BFT.

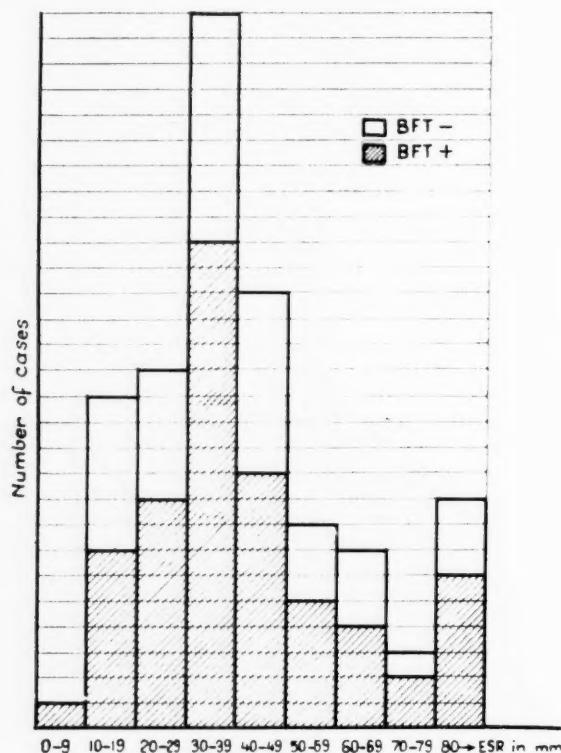


TABLE 1. The correlation between BFT and ESR
(100 cases)

As Table 1 shows, there was no correlation between the BFT and the erythrocyte sedimentation rate (ESR).

The correlation between the BFT and the duration of rheumatoid disease is shown in Table 2. As seen, the duration does not seem to affect the incidence of the positive reaction.

The correlation between the BFT and the Waaler-Rose titer

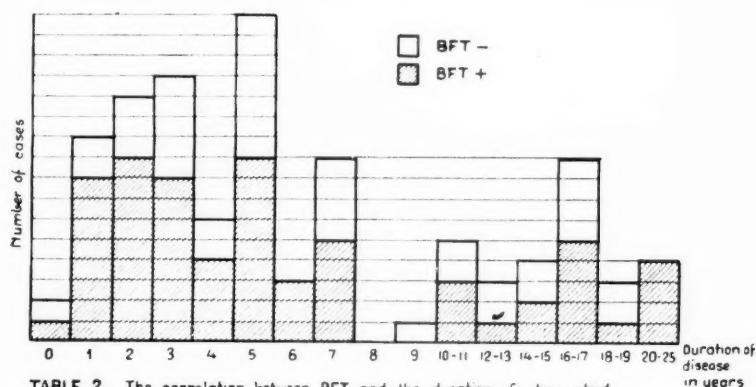


TABLE 2. The correlation between BFT and the duration of rheumatoid arthritis (100 cases)

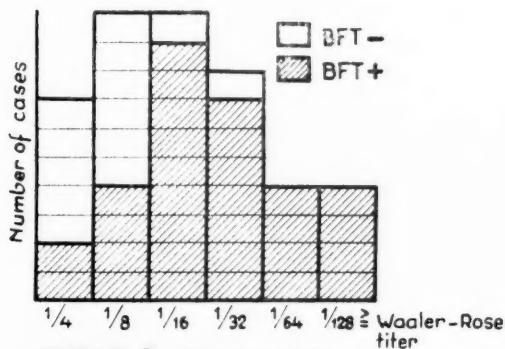


TABLE 3. The correlation between BFT and Waaler-Rose titer (43 cases)

is shown in Table 3. The correlation shows a good agreement when the titer is 1: 16 and upwards. Under this limit the Waaler-Rose titer was estimated as negative. Among these 17 negative cases the BFT was positive in six.

SUMMARY

The BFT was tested in 100 cases of definite rheumatoid arthritis and was found to be positive in 64%, which was less than expected after experiments made in previous investigations. There seems

to be no correlation between the duration of the disease, the sedimentation rate and the stage of the disease. The agreement with the Waaler-Rose titer in 43 cases was not so good as had been expected.

We wish to acknowledge with gratitude the co-operation with Dr. Bunim and his associates at the National Institute of Health, Bethesda, Maryland.

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FROM THE DEPARTMENT OF MEDICAL MICROBIOLOGY, UNIVERSITY OF TURKU

THE OCCURRENCE OF ESCHERICHIA COLI SEROTYPES
26:B6, 55:B5, 86:B7, 111:B4, 125:B15, 126:B16 AND O GROUPS
44 AND 78 IN INFLAMED AND HEALTHY APPENDICES
AND FAECES

by

MATTI K. LEPPÄNEN

(Received for publication November 28, 1958)¹

The etiological significance of certain *Escherichia coli* serotypes in epidemic infantile diarrhoea has been established with a fairly high degree of certainty (1, 4, 6, 21). On the basis of figures reported in the literature up to 1954, «diarrhoeal» *Escherichia* serotypes have been found in the faeces of healthy infants as follows: *E. coli* 111:B4 in 5.7 per cent of 3.603 infants, *E. coli* 55:B5 in 4.1 per cent of 2.436 infants and *E. coli* 26:B6 in 0.9 per cent of 1.189 infants (6). The faecal specimens of noncontact infants examined after 1954 have not been found to contain these *E. coli* serotypes (4, 7). The same serotypes have been isolated from only a small number of adults suffering from diarrhoea (4, 13, 17, 18, 19, 20). Diarrhoeal *E. coli* serotypes have only rarely been found in the faeces of healthy adults, and in these only when they have been in contact with diarrhoeal infants (2, 4, 10, 11, 12, 15, 20). With only two exceptions, these *E. coli* strains have not been isolated from other inflammatory diseases than diarrhoea in humans (6, 16). One exception was a patient with intestinal and meningeal symptoms from whom *E. coli* 111:B4:12 was isolated from both the faeces and cerebrospinal fluid (3) and the other a patient with urinary infection, from whose urine *E. coli* 126:B16 was isolated (8).

The aim of the present study was to determine whether diarrhoeal *E. coli* strains are present in diseased and healthy appendices, in peritoneal exudates and in the faeces of the respective patients.

The bacteriological and serological techniques were those employed by the author in an earlier study (14). The *E. coli* serotypes for which a search was made were the types 26:B6, 55:B5, 86:B7 and 111:B4. In addition, the occurrence of serotypes 44:L74, 125:B15 and 126:B16, which have been encountered in faeces of diarrhoeal infants, and that of strains of O group 78, which have been isolated from calves with white scours, have been determined.

The material examined comprises 257 acutely inflamed and 53 healthy appendices, 40 peritoneal exudates and 278 faecal specimens from the patients in question. The examined 3,500 *E. coli* strains were isolated from various sites as indicated in the following:

257 acutely inflamed appendices	1,893	strains
53 normal appendices	242	"
40 peritoneal exudates	175	"
278 faecal specimens from subjects with acutely inflamed and normal appendices	1,124	"
18 ileocecal lymph nodes	66	"

The ages of the patients from whom the specimens were taken are given in table 1. Six of the patients were two years old. The oldest patient was 74 years of age.

TABLE 1
DISTRIBUTION OF PATIENTS ACCORDING TO AGE

	Age in Years								Total
	2—10	11—20	21—30	31—40	41—50	51—60	over 60		
Patients with acutely inflamed appendices	43	60	62	39	28	20	5		257
Patients with healthy appen- dices	9	17	15	9	1	2	—		53
Total	52	77	77	48	29	22	5		310

RESULTS

None of the *Escherichia coli* strains isolated from acutely inflamed appendices, peritoneal exudates, normal appendices, ileo-coecal lymph nodes, or faecal specimens of patients with normal appendices were of the *E. coli* serotypes that were sought. Two diarrhoeal *E. coli* serotypes were, on the other hand, isolated from faecal specimens of two appendicitis patients. From a four-year-old boy with a typical past history of appendicitis, an appendix was removed which was of phlegmonous type in its histopathological appearance. Of the five *E. coli* strains isolated from the lumen of this appendix, four were of serotype 8:32A:27, but the fifth was not one of the serotypes sought in this study or of the serotypes belonging to O groups 1—25 of the Kauffmann-Knipschmidt-Vahlne antigenic schema. Two of the strains that grew from the faecal specimens of this patient were of serotype 55:B5:4. The boy had not previously suffered from diarrhoea, nor was it established that anyone in his family circle had had this disease. The other patient was a six-year-old boy from whom a gangrenous, perforated appendix was removed and who was found to have diffuse peritonitis. The *E. coli* strains isolated from the appendix lumen and the peritoneal exudate were all of type 2:5L:4. One of the strains isolated from the faecal specimen of the boy was of type 86:B7 and non-motile. The other four strains that grew from the specimen were not of the types sought in this study nor could they be entered into O groups 1—25 of the antigenic schema. Neither did this patient have a past history of diarrhoea. Agglutinins for the serotypes isolated from these two patients were not detected in their sera.

COMMENTS

In common with the earlier studies in which the so-called diarrhoeal *E. coli* strains have been isolated from human infections other than diarrhoea, diarrhoeal serotypes 26:B6, 55:B5, 86:B7 and 111:B4 were not found in the specimens examined in this study except in two cases. Neither were strains of serotypes 125:B15, 126:B16 or 44:L74 or strains of O group 78 found in the acutely inflamed or healthy appendices, peritoneal exudates, ileocoecal lymph nodes, or in the faecal specimens of the patients with normal

appendices. In the case of two boys with appendicitis under seven years of age diarrhoeal *E. coli* strains were isolated from the faecal specimens, but not from the inflamed appendices. This frequency, two out of 278 faecal specimens, conforms with the frequency with which such strains have been previously isolated from faecal specimens of non-diarrhoeal patients who have not been in contact with diarrhoeal patients. This finding thus confirms the view that these serotypes occur only in epidemic infantile diarrhoea.

SUMMARY

Escherichia coli serotypes 26:B6, 55:B5, 86:B7, 111:B4, 125:B15, 126:B16 or strains of O groups 44 and 78 have not been found in acutely inflamed or healthy appendices, in peritoneal exudates, in ileocoecal lymph nodes, or in faecal specimens of patients with healthy appendices. Serotype 55:B5:4 was found in the faeces of an appendicitic four-year-old boy and the serotype 86:B7 in a faecal specimen from a six-year-old boy with perforative appendicitis. Antibodies for homologous *Escherichia* strains were not detected in the sera of these two patients.

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FROM THE DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF TURKU

GLUCURONIDE EXCRETION IN THE ALLOXAN DIABETIC RABBIT

by

W. NIENSTEDT and K. HARTIALA¹

(Received for publication December 10, 1958)

The biochemical mechanism of the glucuronide formation in the organism has been rather well explained. It is evident that the ester type conjugation of suitable substrates with a phenol hydroxyl group is carried through a complex uridine-diphosphate-uridine-diphosphoglucuronic acid cycle (1, 2, 3, 4, 5, 6, 7, 8). The presence and proper function of the energy producing mediators and enzyme catalysts in the cellular components are essential for these reactions. On the other hand these syntheses involve also the availability of glucose in a suitable form (glucose 1-phosphate). The intimate relationship to the carbohydrate metabolism of the glucuronide synthesis stimulated the present investigation. In this work the glucuronide formation from a easily detectable glucurogenic substance, phenolphthalein, was studied under experimental conditions in which the carbohydrate metabolism was impaired by alloxan treatment.

MATERIAL AND METHODS

Prior to the actual experimentation a group of rabbits were subcutaneously given 180—200 mg alloxan per kg body weight. From this treatment 6 rabbits with distinct diabetic syndromes recovered for further use. The diabetes was checked by qualitative urinary sugar (Nylander) and quantitative blood sugar (Hagedorn—Jensen) determinations. Only such rabbits were considered

¹ Supported by a grant from the Sigrid Jusélius Foundation.

diabetic and used in the experiments which showed a strongly positive urine sugar as indicated by a strongly positive Nylander's test and a blood sugar value exceeding 200 mg%. None of the control rabbits had fasting blood sugar values higher than 0.160 mg%. Thus 6 diabetic (4 males and 2 females, mean weight 233 gr) and 7 control rabbits (5 male and 2 female, mean weight 227 gr) were used in the final experiments.

The method of determining the phenolphthalein glucuronide concentration in the urine was a modification of that used by Talalay, Fishman and Huggins (9) for β -glucuronidase activity determinations. 100 mg/kg body weight of phenolphthalein diphosphate (Sigma) was injected subcutaneously. The urine was collected and the sample determinations were made after 3, 6, 12, 18, 24, 36, 48, 72 and 96 hours. Only turnips and water were given to the rabbits during these 4 days. 20 ccm (on the first day) and 40 ccm (on the 2nd to 4th days) of 0.5-N sodium hydroxide was set in the urine collecting bottles in order to prevent the bacterial or enzymatic degradation of phenolphthalein glucuronide. This was found to be more effective than toluene. The collection bottles were covered with a wire mesh which prevented the escape of faeces into the samples. After finishing the collection the samples were neutralized by titration with 0.5—N HCl. The volume of urine was determined by subtracting the volume of the used NaOH and HCl solutions from the volume of the neutralized sample. This sample was then diluted with distilled H₂O so that 1 ccm of the final dilution corresponded 0.1 ccm (samples 1—3), 0.2 ccm (samples 4—6) or 0.5 ccm (samples 7—9) of undiluted urine. 1 ccm of this dilution was pipetted in each of four centrifuge tubes that were then held for 15 min in 100°C water bath in order to destroy all possible enzymatic activity in the urine. After cooling of this 4 ccm of pH 4.5 acetate buffer was added. The buffer added to two of the four tubes contained 1000 units of β -glucuronidase (Worthington)/1 ccm of undiluted urine; no enzyme was added to the two blank tubes. The samples were then held for 24 hours in a 38°C water bath; 5 ccm of pH 11.1 glycine buffer [aminoacetic acid 16.30 gm, sodium chloride 12.65 gm and 11.7 ccm of concentrated NaOH (100 gm of NaOH to 100 ccm of dist. water), made up to 1 liter with dist. water] was then added to each tube. The tubes were centrifuged and the color was determined with a Beckman Spectrophotometer using the

550 m μ wavelength. The value for phenolphthalein glucuronide concentration was obtained by subtracting the mean color reading of the two blank tubes and the mean reading of several control tubes, in which the urine dilution was replaced by corresponding solution of β -glucuronidase in dist. water, from the mean color reading of the actual test tubes. An approximate for the excreted free phenolphthalein was obtained by subtracting the mean color reading of several pure rabbit urine samples from the average reading of the two blank tubes. No significant amounts of free phenolphthalein were excreted after 18 hours. — Values for the calibration linear were obtained by replacing the original urine dilution with known dilutions of pure phenolphthalein glucuronide.

RESULTS AND CONCLUSIONS

The fasting blood sugar values of the alloxan treated animals ranged from 222—395 mg%, the mean being 299 mg%. The corresponding mean in the control animals was 141 mg%. The treatment can be considered to be effective. The other results are graphically illustrated by figure 1. In these curves it can be seen that the administered phenolphthalein is rather rapidly excreted in the urine. The amount of free phenolphthalein is small when compared to the amount excreted in the conjugated form. Half of the total phenolphthalein is eliminated in the urine in 18 hours (diabetic 58, control 56 per cent). In 96 hours the total recovery in the urine of the administered phenolphthalein amounts to 83 ± 11 per cent in the diabetic and 75 ± 8 per cent in the control group.

It is evident from these results that in this respect the alloxan diabetic rabbit does not differ from the normal rabbit.

According to the present concepts the formation of phenolic glucuronides does not proceed through glycolytic processes but so that the carbohydrate part of the conjugate derives directly from glucose (as glucose 1-phosphate) without prior decomposition (11, 12, 13, 14, 15).

It has also been shown that factors such as NaF or iodoacetate which exert their action on the phosphorylation processes inhibit the glucuronide formation (10).

On the other hand Lipschitz and Bueding have found that the glucuronic acid synthesis *in vitro* is augmented by insulin (10).

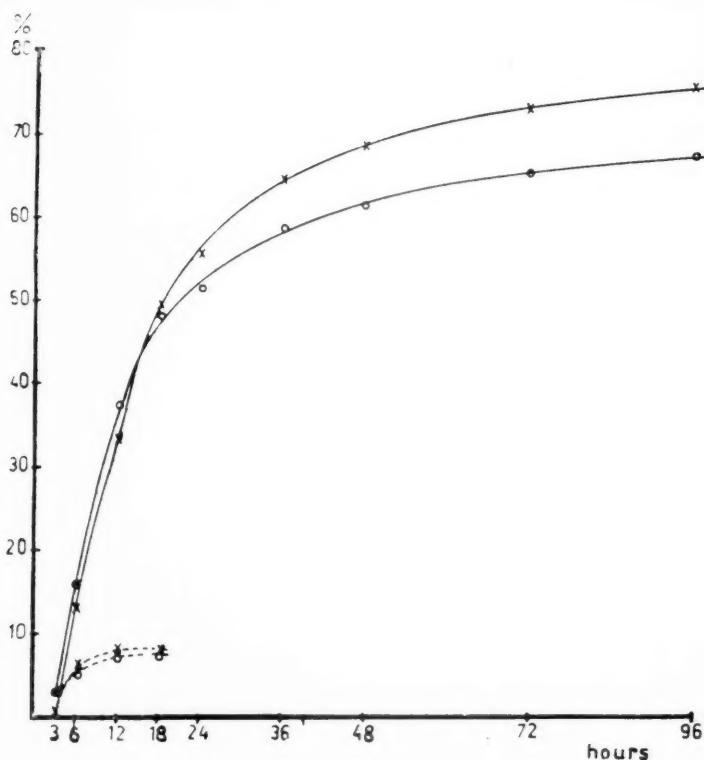


Fig. 1. — Excretion of conjugated (upper curves) and free phenolphthalein (lower curves) in diabetic (X) and control (O) rabbits after subcutaneus administration of 100 mg/kg of phenolphthalein diphosphate. The results are expressed as percentage of the total administered amount of phenolphthalein.

In this type of experimentation as well as in alloxan diabetic animals the actual enzymatic breakdown of glucose is not effected, it is only the availability of glucose for the cells which is effected. The markable parallelism of the glucuronide excretion found in the present material allows the conclusion that the usual difficulties met in the utilization of glucose in diabetic conditions and which lead to an elevation of the blood sugar contents do not manifest themselves in the glucuronide conjugation processes of the whole organism.

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FROM THE DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF TURKU

FLUORESCENCE OF TETRACYCLIN IN EXPERIMENTAL ULCERS AND REGENERATING TISSUE INJURIES

by

ILKKA HÄKKINEN and K. HARTIALA

(Received for publication October 28, 1958)

The yellow fluorescent substance which is formed in the organism after the administration of tetracyclin (14, 13) has been studied more exactly by spectrophotometric and chromatographic means and it has been found that this material giving the fluorescence is tetracyclin itself possibly conjugated with a metallic cation to a organic matrix (16). It has also been found by Loo *et al.* (11) that one part of the fluorescent material remaining in the surrounding of mice sarcoma is pure tetracyclin and it has been assumed that it forms some kind of a loose complex compound with a peptide- like substance specific just for sarcoma tissue. This later statement has not been verified more exactly.

Our interest in the subject was aroused by the observation on the similar kind of reaction exerted by two entirely different tissues, the bone and tumour, upon tetracyclin administration. In the experiments performed by Häkkinen (10) it became evident that the fluorescent tetracyclin becomes fixed also into certain soft tissues when the organism has received excessive amounts of dihydrotachysterol and particularly in regions where calcium metastases were histologically present. Similar changes have been produced with excess administration of parathormone. Since the changes caused by the excessive doses is in some way traumatic (1, 2, 12), it occurred to be worth while to study the behaviour of tetracyclin also in connection of other kinds of tissue injuries and

particularly in experimental ulcer conditions. In the attempts to throw light into retaining of the fluorescence in tumour tissue partial hepatectomy was performed in rats and observations were made on the fluorescence phenomena during the regeneration period. It is known that by these means a lively mitose period is established (3, 4, 8) as is the case in tumour growth.

MATERIAL AND METHODS

10 dogs of both sex, weight range between 8—12 kg. and age 6 months to 2 years, were fed cinchophen (Merck) with a gastric tube the daily dose being 200 mg/kg body weight (17).

The administration of cinchophen was continued over a period of 12 days. The diet was free and water was given ad libitum. On the 11th day each dog received intramuscularly Achromycin (50 mg/kg) in a single dose. The dogs were sacrificed on the 14th day from the beginning of the cinchophen feeding, the stomachs were cut open and inspected in ultraviolet light (3660 Å, die analysen Lampe, Original Hanau). Histological specimens were taken from the detected ulcers, these were fixed in formalin and stained according to the van Gieson procedure. Additional specimens were taken from the gastric mucosa, submucosa and muscular layer as well as from the fluorescent tissue for later chemical Analyses. The femur bone was also excised in each dog for detection of the tetracycline fluorescence in the bone. 10 male guinea pigs (300—400 g) were orally given a 5 cc single dose of 60 per cent alcohol similarly to the method described by Williams (18). Food and liquid were allowed ad libitum. 5 hours after the alcohol treatment the guinea pigs were injected intramuscularly with 50 mg/kg of Achromycin. The animals were sacrificed 1½—2 days after the beginning of the alcohol application. The stomachs were excised and inspected in UV light.

10 male rats (Wistar) wt.c. 200 g, were given a single oral dose of 3.5 cc of 60 per cent alcohol, food and liquid ad libitum. Tetracycline was given as to the guinea pigs and the animals killed again 1½—2 days after the alcohol treatment. The stomachs were inspected in UV light.

On 10 male rats (Wistar), weight c. 200 g, was performed the pylorus ligation according to Shay *et al.* (15), 50 mg/kg of tetracy-

cyclin was injected to the animals 4 hours after the operation and the rats were kept alive about 1½ days after the operation by giving subcutaneous saline-glucose infusions. The stomachs were cut open and studied in UV-light.

23 female rats (Wistar) wt. c. 200 g were operated under aether anaesthesia at which two thirds of two hepatic lobes were removed through a mid-line excision. The surface of the liver as well as the abdominal wall were closed with silk. The skin was closed with aggraphs pins. The rats were divided into groups 3 animals in each. They received 50 mg/kg of tetracyclin as a single injection 1—5 days before their sacrifice. The animals were killed in groups in regular intervals 1—14 days after the operation, they were cut open and inspected in ultraviolet light. Histological specimens were taken from the fluorescent parts, these were fixed in formalin and stained with hematoxylin and eosin.

RESULTS

In all dogs a penetrating histologically clear ulcer developed in the pyloric region. The diameter of these ulcers was 0.5—1.5 cm. Several smaller superficial erosions were yet found in some of the dogs. The dogs lost much weight at the end of the cinchophen treatment, vomited and lost their appetite. In the UV-inspection a bright yellow fluorescence was found, this reflected as an almost continuos 0.5—1 mm thick layer through the margin of the ulcer and the bottom of the callus formation. The superficial erosions showed also a distinct fluorescence. The fluorescence phenonen revealed the pyloric ulcers significantly clearer than in usual light (Fig. 1).

In the upper two thirds of the stomachs of the alcohol treated guinea pigs hemorrhages and occasionally large ulcers were also found, which extended to the muscular layer. An intensive yellow fluorescence was present on the margins of the ulcers but not in the bottom. The hemorrhagic regions did not show a fluorescence. In two of the alcohol treated rats distinct ulcers had been produced, in the others there was found only mucosal hemorrhages of varying degree. The rat ulcers were on the glandular part of the stomach and a heavy yellow fluorescence was present on the edges of them. In the Shay rats typical ulcers had developed on the squamous



Fig. 1. — The fluorescence of tetracycline in experimental ulcers and regenerating tissue injuries.

Photograph taken from the pyloric region of the dog stomach in ultraviolet light (Die Analysen Lampe, Original Hanau). The large crater-like cinchophen ulcer is easily visable. On the basis of the ulcer as well as here and there in the mucosal erosions of the pyloric region are seen as bright strongly illuminated points the fluorescence phenomen caused by tetracyclin.

epithelial parts in the stomach, these were always multiple. No fluorescent regions in the stomach were found. This can not be due to circulatory disturbances provoked by the pylorus ligation since greater gastric blood vessels were not ligated.

The rats, on which partial hepatectomy was performed, were incised and the surroundings of the traumatic area in the abdominal wall as well as the hepatic incision scar were inspected in ultraviolet light. In the rats killed 1—3 days after the operation a marked yellow fluorescence was found in the traumatic areas of the abdominal wall. Specially marked was this in the site of the silk sutures, which increased up to 1—3 days in order to disappear thereafter completely in the animals killed after this. In the excision scar of the liver a constant fluorescence was noted, this being concentrated, however, only around the silk sutures and was not present in the

histologically typical regeneration regions. The fluorescent tissue appeared to be partly a necrotized mass surrounded by a infiltration cell zone without signs of regenerative parenchym. The fluorescent region in the liver appeared to show signs of an increase in the animals killed 1—3 days after the operation, between the 3—14 postoperative days it remained almost unchanged.

DISCUSSION

According to Williams (18) the lesions produced with alcohol in the gastric mucous membrane in guinea pigs as well in rats must be considered as acute traumatic injuries different from the actual ulcers caused e.g. by cinchophen in dog. Nevertheless the ulcer is a tissue injury which has the tendency for healing. The localisation of the fluorescence around the described traumatic region was a constant phenomena, which perhaps could be explained on the basis of the change in the polymerization degree of the ground substance surrounding the injurious tissue and by the formation of active groups in the carbohydrate protein complex of the ground substance. In the preliminary experiments (in preparation) the fluorescent tissue of the ulcers has contained more mucoproteins than the other parts of the stomach. The complete failure to produce the fluorescence in Shay-rat ulcers remains still without any explanation. Dumphy and Udupa (6) have studied the healing of traumatic lesions and observed that the mucopolysaccharide contents in the serum increased abruptly 0—3 days after the operation in order to decline thereafter just as fast. The fluorescence caused by tetracyclin in the abdominal wall of the operated rats appears to follow the same scheme.

The regenerative liver and the tumour tissue hardly have much common although both are characterized by rich mitosis. The fixation of tetracyclin and the phenomena associated with the mitotic process do not appear to have much to do with each other. For advancement of this idea has the author performed some experiments with tissue cultures (in preparation).

SUMMARY

The cinchophen ulcer as well as the alcohol induced ulcers in guinea pig and rat were revealed to give a yellow fluorescence after the intramuscular administration of tetracycline. The Shay-ulcers did not show a similar fluorescence. In partially hepatectomized rats the tetracycline fluorescence was present in the necrotic regions of the hepatic tissue and around the silk sutures. Also in the abdominal wall layers of the same animals the fluorescence was clearly present in the incision scars 1—3 days after the operation.

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FROM THE RESEARCH LABORATORIES OF THE STATE ALCOHOL MONOPOLY,
HELSINKI, AND THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY,
UNIVERSITY OF HELSINKI

EFFECT OF ETHYL ALCOHOL ON THE SUSCEPTIBILITY OF MICE TO STAPHYLOCOCCAL INFECTION

II

by

O. WASZ-HÖCKERT, TIMO KOSUNEN, and J. KOHONEN

(Received for publication November 14, 1958)

In a previous paper (2) we reported the results of an investigation concerning the effect of acute alcohol consumption on the resistance of the mice to staphylococcal infection. Eight per cent ethanol significantly shortened the survival time. Using a bacterial enumeration technique, the number of viable staphylococci in the liver was shown to be significantly higher in the ethanol-consuming mice than in the control mice.

The present study deals with the effect of a longterm alcohol intake, before and during the infection.

MATERIAL AND METHODS

Mice. — One hundred and five Swiss Albino Webster mice, ranging in weight from 19 to 24 g at the time of inoculation, were used.

Bacterial Culture. — *Staphylococcus aureus*, strain »Orion», cultured for 24 hours in broth, was used diluted with physiological saline in the ratio 1 to 2. The challenge dose of 0.2 ml was injected intravenously in one of the tail veins.

Aided by grants from the Finnish Foundation for Alcohol Studies.

TABLE
GAIN IN WEIGHT

Group	Age in		
	4	5	6
Mean weight in grams			
Ethanol group	14.9 ± 1.0	17.9 ± 1.3	20.0 ± 1.9
First control group	15.0 ± 1.0	18.0 ± 1.3	19.9 ± 1.7
Second control group	15.0 ± 0.9	17.9 ± 1.2	19.9 ± 1.6

Ethanol. — Five per cent w/w ethanol-water solution was used as drinking fluid for the mice in the experimental and the second control group.

Technique. — The technique employed involved noting the survival time, measuring the gain in weight of the mice and making a bacterial enumeration. The quantitative method of bacterial enumeration was described in detail previously (1).

PLAN OF THE EXPERIMENT

The mice were kept in wire-mesh-bottom cages. Temperature and humidity in the room were controlled. The temperature during the experiment was about 24°C and humidity 65 to 70 per cent. The mice were kept 10 to a cage, except for the second control group in which all 15 mice were in one cage. Food pellets of a standardised composition were available *ad libitum*. The drinking fluid was offered from inverted graduated bottles.

In the beginning of the experiment the mice were four weeks old, weighing from 13 to 16 g. They were divided into three groups and treated as follows:

Ethanol Group. — The number of mice in this group was fifty. The only drinking fluid for these mice was the 5 per cent aqueous solution of ethanol. The mice were challenged when seven weeks old, *i.e.* three weeks from the beginning of the experiment.

First Control Group. — This group consisted of forty mice. For these mice the only drinking fluid was water. They were challenged at the same time as the mice of the ethanol group.

Second Control Group. — A group of 15 mice was not challenged, but given ethanol as in the ethanol group.

Records were kept concerning the survival of the mice. For

1
OF MICE

Weeks	7	8	9	10	11
\pm standard Deviation					
	22.4 \pm 2.0	23.2 \pm 2.2	25.5 \pm 2.5	26.5 \pm 2.7	27.0 \pm 2.7
	22.4 \pm 1.9	23.3 \pm 2.0	25.4 \pm 2.1	26.6 \pm 2.2	27.2 \pm 2.5
	22.3 \pm 1.9	23.3 \pm 1.9	25.6 \pm 2.1	26.9 \pm 2.1	27.3 \pm 2.3

bacterial enumeration study four mice per challenged group were withdrawn by random sampling at intervals as shown in the figures. The mice were sacrificed, the spleen, kidneys and liver removed by sterile technique, homogenised, and the number of viable bacteria per ml of tissue determined.

RESULTS

Ethanol in the doses used did not alter the behaviour of the mice, nor were there any detectable amounts of ethanol in their blood, determined by the Widmark method.

Body Weight. — The gain in weight was similar in all the three groups (Table 1).

Survival. — Mortality was very low, 1—2 per challenged group, thus there was no difference in survival time.

Bacterial Enumeration. — The number of viable bacteria in the organs was determined during the experiment. The results are shown in Figs. 1—3. The bacterial population curves in the kidneys (Fig. 1) and spleen (Fig. 2) showed no difference between the groups. This and the course of the curves were very similar in our study of acute alcohol consumption (2).

TABLE 2
STAPHYLOCOCCAL INFECTION IN THE LIVER

Days after Inoculation	Number of Animals	Alcohol Group		Control Group		χ^2	P <
		Infected	Non-Infected	Infected	Non-Infected		
1—4	32	16	0	15	1	1.032	0.500
7—30	54	13	15	4	22	6.023	0.025
1—30	78	25	15	15	23	4.136	0.050

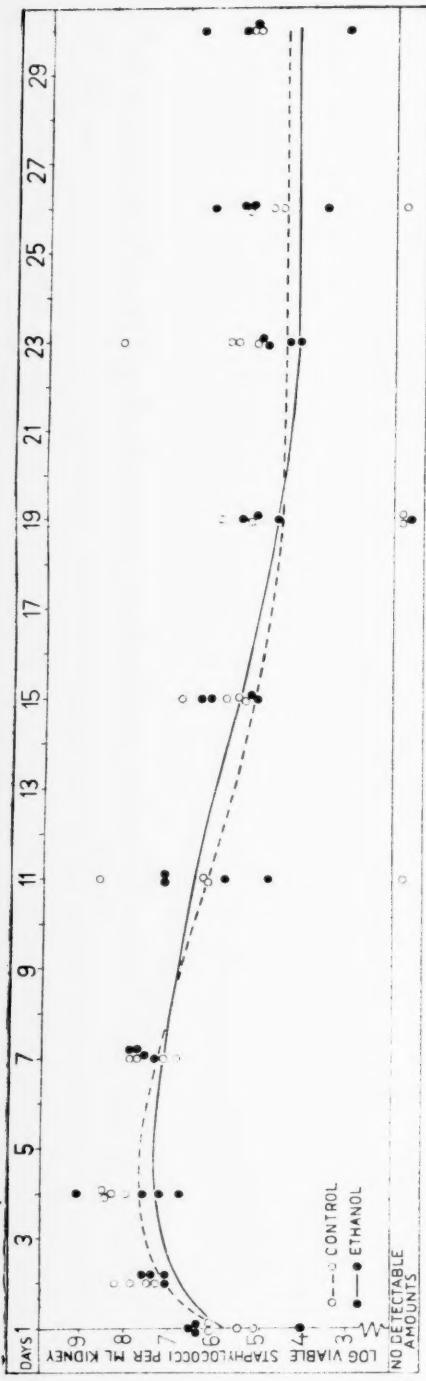
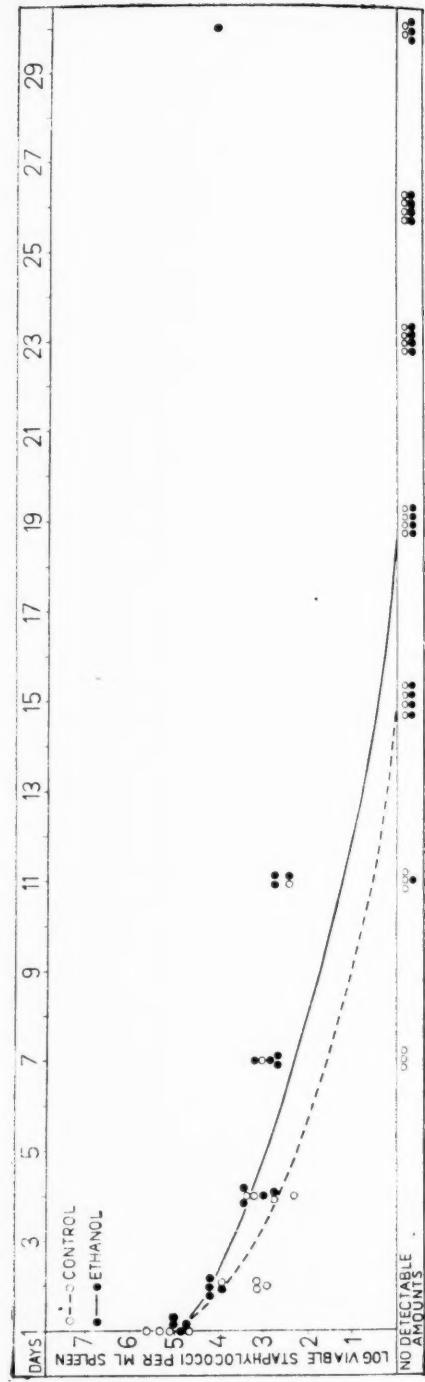


Fig. 1.



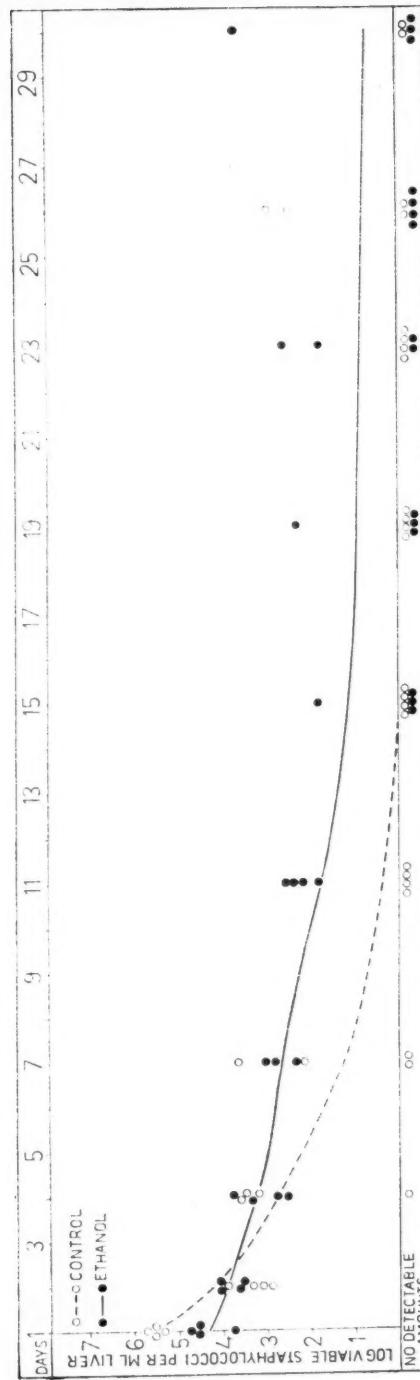


Fig. 3.

In the liver (Fig. 3), however, statistically significant differences were seen one week after the challenge, as Table 2 reveals. This result for the liver concurs with the findings of the experiment mentioned above (2).

DISCUSSION

Our object was to study the effect of ethyl alcohol on the resistance of the host organism to infection. A similar investigation has been previously reported by us (2). There are two main points of difference between our both experiments: (a) in the first part (I) the alcohol consumption was more acute, in the later experiment (II) more chronic; and (b) the alcohol concentration was lower in the later experiment. In both experiments we tried to reproduce in some degree the corresponding human conditions.

The difference between survival times in the two experiments was astonishing. In experiment I the difference in survival time between the groups was highly significant, in II there was practically no mortality at all. This could be due at least partly to the difference in the alcohol concentrations.

With the bacterial enumeration technique used no differences were seen in the spleen and the kidneys between the ethanol and non-ethanol groups. Contrary to this, a difference in the liver was seen in both tests, although this difference was not so marked in the later experiment. Possibly the function of the liver as a detoxifying organ is responsible for the phenomenon observed. The distribution of the bacteria in the organs studied and the changes in the population curves reflect physiological and functional properties of organs.

Possible explanations of these differences could be immunological reactions, metabolic disturbances affected by alcohol or the lowered protein intake in the alcohol group. It is evident, however, that the acute or chronic consumption of alcohol by mice disturbs the host-parasite relationship by lowering the resistance to staphylococcal infections.

SUMMARY

The effect of ethyl alcohol on the resistance of the host to staphylococcal infection is studied in two experiments. In the first experiment (2), comprising 135 mice, the alcohol consumption was more acute. The mice received nothing but eight per cent ethyl alcohol as drink after being challenged. In the present experiment the five per cent alcohol intake of the 105 mice was longterm being started 3 weeks before the infection.

In the alcohol consuming group the survival time was decreased only in the first experiment but the number of viable staphylococci in the liver increased during the observation time of three weeks in both experiments. By the same bacterial enumeration technique no differences were observed in the spleen and kidneys.

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OXYDATION OF *p*-PHENYLENEDIAMINE BY UMBILICAL CORD EXTRACT

by

EINO KULONEN

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This report is motivated by an earlier observation that the oxydation of *p*-phenylenediamine in the presence of H_2O_2 was catalyzed by heated umbilical cord extracts and that this ability was decreased when the hyaluronic acid in the extract had been depolymerized with hyaluronidase (2). The catalyzing ability was not present in a solution of chemically pure hyaluronate. In view of the clinical interest on the oxydation of *p*-phenylenediamine by plasma (1), the phenomenon was studied further. Evidence is presented below that the oxydative capacity is attached to the protein of the extracts. The presence of the hemin cannot be excluded and it seems a quite plausible catalyst, since proteins in general do not react.

Glucose (to represent the reducing substances produced in the enzymatic hydrolysis), did not affect the reaction. However, when hematin was heated with glucose solution at water bath, the catalysis was appreciably retarded but not prevented.

EXPERIMENTAL

Preparative. — The ground umbilical cords were extracted with 0.2 M disodium phosphate solution for several hours in the room temperature. The viscous extract was cleared by filtration and the supernatant dialyzed for several days against tap water. The precipitate was discarded and the yellow solution used as the crude extract. It contained 1.6 mg. nitrogen and 1.5 mg. glucosamine per ml.

Fractionation in the silica gel electrophoresis was made in a trough 8 cm. broad and 0.5 cm. deep. The electrolyte was pH 6.8 phosphate buffer (*M/10*). The voltage gradient was about 15 V./cm. and the time 3 hours. The fractions (1 cm. broad) were cut and eluted with water. The analyses of nitrogen and glucosamine were carried out from each fraction.

For the *ethanol fractionation* the original crude extract was treated with commercial trypsin and the digest dialyzed against tap water. To 50 ml. of fluid 0.122 g. BaCl₂ was added and pH adjusted to 6.4. Cold absolute ethanol was added to 10% (v/v) concentration, the precipitate collected in centrifuge and dissolved into 50 ml. of water. To the supernatant more ethanol was added in calculated portions and the precipitates collected at 10% (v/v ethanol) intervals and dissolved into 50 ml. water each. Nitrogen and glucosamine were determined.

Testing. — In a series of test tubes following solutions were pipetted: 0.2 ml. of umbilical cord extract, 0.2 ml. of 1% fresh aqueous solution of *p*-phenylenediamine (Merck), 0.2 ml. of 3% hydrogen peroxide solution, 1 ml. of buffer (usually pH 8.6 bicarbonate, *M/5*) and 5 ml. water. The colour was measured after 1 hour intervals at wavelength of 5000 Å. When the effect of the different additions was studied, water was used to adjust the final volumes.

RESULTS

The effect of pH is shown in Fig. 1. Afterwards all the following experiments were made using pH 8.6 bicarbonate buffer. The amount of the hydrogen peroxide was not critical (Fig. 2). The addition of glucose did not affect the catalysis.

Fig. 3 shows the catalyzing capacity of the different electro-phoretically obtained fractions and Fig. 4 of ethanol-precipitated fractions.

The mentioned effect of hyaluronidase could be observed but was small.

DISCUSSION

The question presents itself how to explain the original dependence on the degree of polymerization. The catalyzing action was presumably due to hemoglobin derivatives, which were not completely removed. When the samples were heated to inactivate the enzyme, a complex might have been formed between the breakdown products and hemoglobin derivatives, which were then less active in catalysis. This explanation is supported by

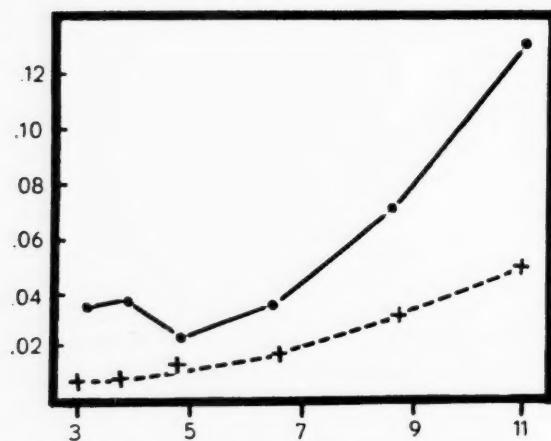


Fig. 1. — Effect of pH on the intensity of the oxydation. Solid line with umbilical cord extract, dotted without (abscissa: pH).

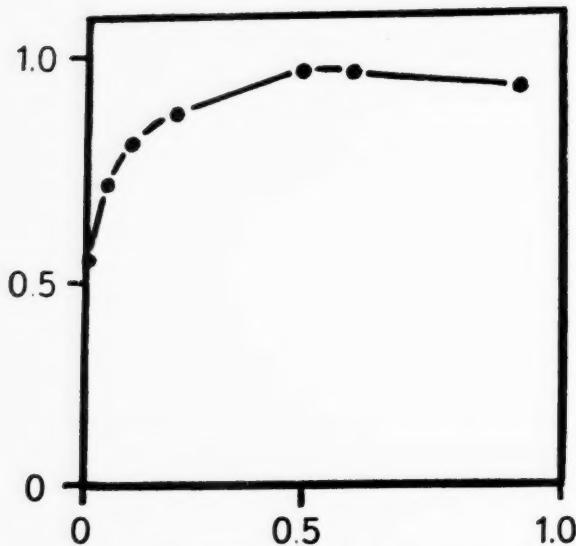


Fig. 2. — Effect of different amounts of hydrogen peroxide on the oxydation (abscissa ml. of 3% H₂O₂).

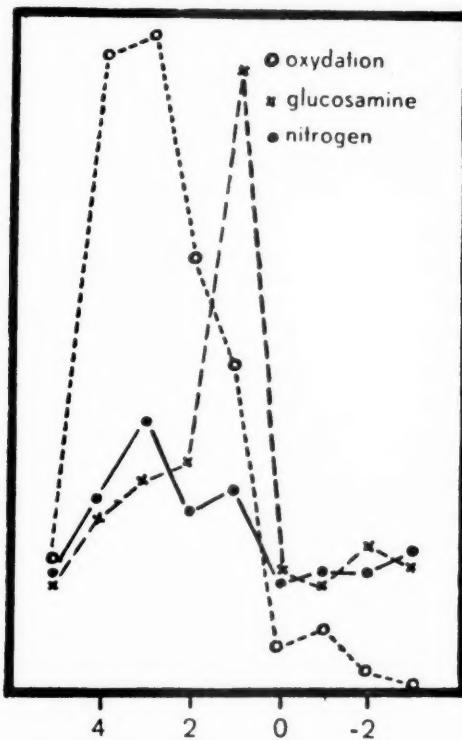


Fig. 3.—Effect of different electrophoretically separated fractions of umbilical cord jelly on the intensity of oxydation. (The abscissa refers to the distance of migration in cm.)

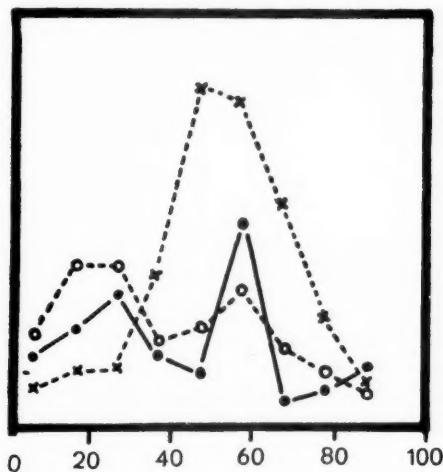


Fig. 4.—(The symbols as in Fig. 3) The effect of umbilical cord preparations obtained with stepwise ethanol fractionation. (The abscissa refers to the percentage of ethanol.)

model experiments. The heating of crude enzymatic hydrolysate may cause the formation of other artefacts also, e.g. the »oxime-like» products described earlier (2).

SUMMARY

The ability of the umbilical cord extract to catalyze the oxydation of *p*-phenylenediamine is traced to protein fraction, possibly to hemoglobin derivatives.

Acknowledgment. — The skilful assistance by Miss Ritva Sellge and the support from the Sigrid Jusélius Foundation are gratefully acknowledged.

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FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF HELSINKI

THE CONVULSIONS AND THE BRAIN ACETYLCHOLINE METABOLISM AFTER MORPHINE AND NALORPHINE

by

P. LAVIKAINEN and M. MATTHILA

(Received for publication January 5, 1959)

INTRODUCTION

The convulsions induced by morphine are considered to be of central origin. They may follow the stimulation of some brain centres and/or the depression of inhibitory centres. Central analeptics enhance morphine convulsions (11). Nalorphine, an antagonist of morphine causes contrary changes in EEG to those produced by morphine (4). Nalorphine is not capable of preventing the euphoria and smooth muscle spasms caused by morphine, though it diminishes the depressive effects on respiration and consciousness.

Morphine inhibits cholinesterase (ChE) (2, 7) and the liberation of brain acetylcholine (ACh) *in vitro* (10). Some morphine antagonists also act in the same way (7, 14). During morphine analgesia the ACh content of the brain is elevated and the antagonistic levallorphan does not prevent this elevation (7). The potentiating action of the anticholinesterasic acting physostigmine and neostigmine on morphine analgesia is undetermined (7).

Our purpose was to study the action of nalorphine on morphine convulsions of the rabbit and the action of morphine and nalorphine on the ChE activity in the brain and spinal cord and the liberation of brain ACh *in vivo*.

MATERIAL AND METHODS

Gray rabbits weighing 2000—3000 g and white rats weighing 150—200 g were used.

The rabbits were injected subcutaneously with morphine chloride in 3 per cent water solution, the rats intraperitoneally. Nalorphine bromide (Burroughs Wellcome)¹ was injected into the ear veins of the rabbits in 4 per cent water solution. The rats received the same solution intraperitoneally.

The convulsions of the rabbit were studied inspected. More precisely the gastrocnemius contractions were recorded via the cut Achilles tendon (8). The trunk and limbs of the rabbit were fixed on the table without pressing the breathing muscles and the gastrocnemius. The contractions were recorded and traced (enlarged 4 times), a counterweight of 30 g being used.

The rats were decapitated 50—60 minutes after morphine injections and 15—20 minutes after nalorphine injections. The brain was prepared and cooled for 15 minutes at —16°C. The brain was cleft and from one half the ACh liberation was determined and the ChE activity from the other. The cervical and thoracal parts of the spinal cord were prepared and preserved at —16°C for a maximum period of one week before the assay.

ACh liberation was determined by means of the whole cell method of Trautner and Messner (16). The composition of the incubation fluid was as follows (mM/l): NaCl 94, KCl 26, NaHCO₃ 23, MgSO₄ 0.4, KHPO₄ 0.4, CaCl₂ 1.4, glucose 5 and eserine salicylate 0.2. Brain mince and incubation fluid in the proportion of 1: 20 were placed in a Warburg vessel; the brain mince was teased into fine strands of 2—3 mm in length, the contents of the cyvette was aerated with a gas mixture of O₂ (95 per cent) and CO₂ (5 per cent) and then incubated and shaken in a Warburg thermostat at 37.5°C for 90 minutes. After the incubation, the pH was adjusted at 4 with 5 drops of 0.1—N HCl. The suspension was centrifuged and the supernatant fluid decanted. The precipitate was washed twice with 0.5 ml of incubation fluid and fluids were added to the supernatant fluid. The «bound» ACh was liberated by cooking the precipitate in the pH 4 for 20 minutes. Just before the assay, the samples were neutralized with 0.1—N NaOH, using 2 drops of bromthymolblue (2 per cent) as indicator and then diluted with water ten times. The ACh was assayed with the leech dorsal muscle. The standard solution (in dilution 10⁻⁷) was prepared from ACh chloride (Hoffmann-La Roche) using incubation fluid as solvent, and then diluted ten times with water as a sample. The brain extract that was cooked in alkaline and then neutralized caused no contractions.

ChE activity was determined by the manometric method of Warburg as modified by Augustinsson (1). Acetyl-beta-methylcholine (MeCh) was enzymatically hydrolyzed in bicarbonate buffer and the liberated

¹ We are grateful to Burroughs Wellcome & Co. for the nalorphine bromide samples.

CO_2 was measured manometrically. The composition of the buffer solution was as follows (per cent w/v): 0.9 NaCl 100 ml, 1.26 NaHCO_3 30 ml, 1.20 KCl 2 ml and 1.76 MgCl_2 2 ml. 0.3 ml of 0.3-M MeCh was pipetted into the side bulb of the vessel and the final concentration of brain suspension in buffer solution was 1: 100, the total volume being 3 ml.

TABLE 1

THE ACTION OF MORPHINE AND NALORPHINE ON THE CONVULSIONS OF THE RABBIT

Number of Rabbit	Effect of Morphine			Effect of Nalorphine			Lethal
	Dose of Morph. mg/kg	Convulsions		Hours after Morph. When Inj.	Dose of Nalorph. mg/kg	H. after Inj. When Began	
1	25	—					
2	50	—					
3	100	—					
4	110	2.00	++				
5	120	—					
1	135	—					
2	140	—					
6 ¹	150	1.20	+++				+
7	200	0.50	+++				+
5	160	—					
4	130	1.10	+++	1.35	10	—	
8	210	2.00	+++	2.40	2	+++	+
1	140	2.30	++	3.30	2	+	
9	130	1.25	+++				
9	145	1.15	+++	2.40	10	+++	+
2	160	1.30	++	2.00	5	+	
10	210	3.05	+++	3.15	8	+++	+
4	135	1.00	+++	1.40	14	+++	+
2	150	1.40	++	2.20	20	—	
11				40	—		
11				80	—		
12				150	—		
13				250	0.30	+++	
				1.15 ²	240	1.45	+++
							+

¹ = received CaCl_2 50 mg/kg.

² = the rabbit recovered and was quiet before the reinjection.

RESULTS

A. Convulsions of the Rabbit. — The smallest doses of morphine that induced convulsions were determined. The results are set out in Table 1. The degree of the strength of the convulsions is expressed by + markings. Opisthotonus is indicated by +++.

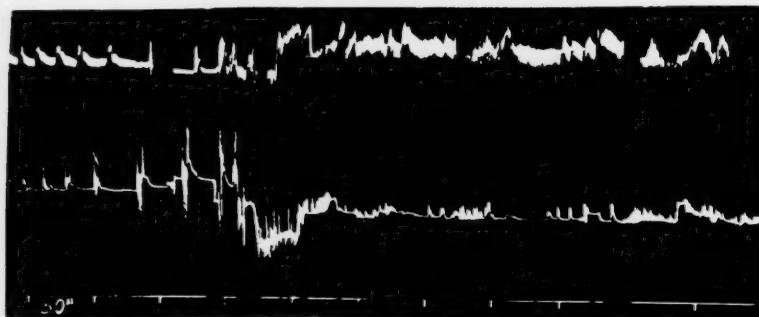


Fig. 1. — Morphine 200 mg/kg. Convulsions after 50 minutes. Death after 2 hours. The upper curve depicts breathing, the lower curve gastrocnemius convulsions. Picture of final stage.

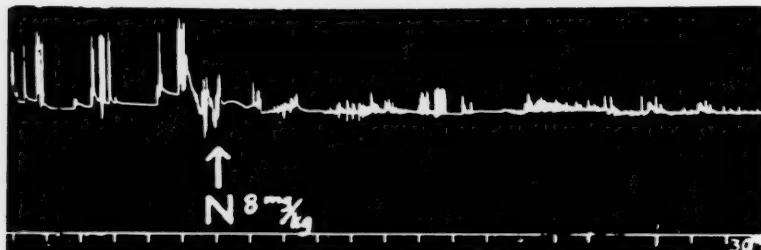


Fig. 2. — Morphine 210 mg/kg. Convulsions after 2 hours. Nalorphine 8 mg/kg at 2 hr. 15 min. point. Animal lived a further 10 minutes.

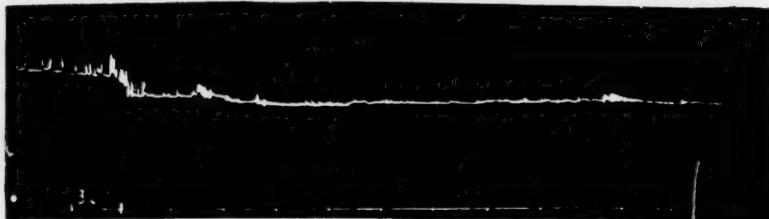


Fig. 3. — Nalorphine 250 mg/kg. Rabbit got convulsions, recovered from them fully, was given 240 mg/kg nalorphine one hour later, convulsions them occurred which proved lethal.

Small doses of nalorphine were given to the rabbit at various intervals after morphine injections. The action of nalorphine is shown in Table 1. The rapid respiration of the rabbit in convulsions increased and the animal showed passing symptoms of waking. Nalorphine was not capable of preventing the strongest convulsions and the death of the animal (figure 2). Convulsions also change to tremor without nalorphine (figure 1), but nalorphine acted more dramatically and quickened the death of the animal.

The doses of nalorphine causing death and convulsions were determined. The results can be seen in Table 1. Strong excitation, miosis, rapid and strong breathing and restless movements preceeded the convulsions. These were not strongest in the limbs as is the case with morphine but in the trunk and above all in the breathing muscles. The convulsions were continuous tremors (figure 3).

B. Enzyme Studies. — Samples of four rats were studied simultaneously. One rat received morphine 50 mg/kg (M_1), one morphine 400 mg/kg (M_2), one nalorphine 500 mg/kg(N) and the fourth acted as a control. The clinical symptoms in group M_1 were practically normal respiration, excitation, rigidity and analgesia. The respiration in group M_2 was very depressed and the animals were under deep anesthesia. The rats in group N breathed forcefully and excitation, tremor and convulsions were observed before decapitation. In some cases the brain was divided into forebrain and hindbrain medulla oblongata included, via the cranial edge of the pons. The amounts of liberated ACh are shown in Table 2 expressed as ACh chloride.

More ACh was liberated from the brain of M_1 rats than from the brain of M_2 rats. The differences are clearly apparent in experiments 7 and 8. M_1 administration did not prevent ACh liberation in any experiment. The N results are distributed on both sides of the control results. ACh liberation in M_2 rats was on an average less than normal and in them the »bound» ACh was a little greater, but these differences are not significant.

The mean ChE activity in the brain and spinal cord can be seen in Table 3. Some assays were carried out using butyrylcholine as substrate. The activity was less and differences as small as when using MeCh. On the average ChE activity follows ACh liberation. The differences are not significant. ChE activity in the spinal cord was roughly as great and the differences as small as in the brain.

TABLE 2
LIBERATION OF BRAIN ACETYLCHOLINE ($\mu\text{g/g/h}$) EXPRESSED AS ACETYLCHOLINE CHLORIDE

	Total Brain							Forebrain		Hind-brain		"Bound" ACh in Total Brain		
	1	2	3	4	5	6	Mean	7	8	7	8	1	2	5
K	12.8	14.4	13.6	10.1	6.8	9.2	11.1	24.2	16.8	4.0	6.0	1.6	1.3	1.8
M ₁	15.2	7.2	18.0	9.2	12.0	16.0	12.8	36.0	16.8	37.4	5.8	1.8	1.3	1.8
M ₂	9.2	2.4	8.0	8.0	8.1	9.3	7.5	10.8	5.4	1.8	3.6	1.9	1.6	1.9
N	16.6	18.4	24.0	6.0	3.6	4.6	12.3	20.2	13.8	46.4	54.2	1.6	1.4	2.0

Notes: ¹ M₁ = 75 mg/kg. ² M₂ = 500 mg/kg. ³ M₁ = 60 mg/kg.
⁴ N = 400 mg/kg. ⁵ N = 300 mg/kg.

TABLE 3
CHOLINESTERASE ACTIVITY IN THE BRAIN AND SPINAL CORD OF THE RAT
(LIBERATED CO₂ $\mu\text{l/g}/30 \text{ min.}$)

	Brain							Spinal Cord						
	1	2	3	4	5	6	Mean	1	2	3	4	5	6	Mean
K	2633	2031	1734	2450	2080	2057	2167	1955	2045	2180	2188	2020	2077	
M ₁	2233	2596	2109	2576	2251	2455	2370	1848	2384	2215	2230	2216	2179	
M ₂	2328	2088	1599	2802	1696	2350	2132	2297	1142	2085	2330	2164	2003	
N	2533	2734	2216	2431	2494	1845	2375	1985	2221	1344	2412	2228	2238	

DISCUSSION

Morphine convulsions of the rabbit were not eliminated or decreased by CaCl₂ and consequently they are not caused by hyperventilation and alkalosis. The effect of nalorphine was not beneficial if the morphine dose was sufficiently great. On the contrary it quickened the death of the animals. This condition after lethal doses of morphine may be regarded as an irreversible enzymatic disorder. The action of nalorphine on morphine convulsions may be explained by the fact that the very great dose of morphine also stimulates such brain centres as it inhibits in lesser doses and which nalorphine stimulates (4).

The lethal dose of nalorphine (50 mg/kg) for the rabbit determined by Unna (17) is too small. Our results correspond better with those obtained in experiments on mice (6, 17), in which the lethal dose of nalorphine was a little greater than that of morphine.

On the basis of changes in the brain content of ACh has been supposed that excitation corresponds with an increased ACh synthesis and anesthesia with a decreased (12, 9, 13). Morphine prevents the liberation of brain ACh *in vitro* but does not effect ACh synthesis by cell free brain extracts (10). Our experiments do not completely tally with as the results of some other investigators. Like them, we observed ACh liberation in the hindbrain to be less than in the forebrain (5). Small doses of morphine did not prevent ACh liberation in the brain. This also tallies with previous investigations (10). But in our experiments great doses did not markedly prevent ACh liberation either. The different in the results obtained by us may be due to many causes:

1) Individual differences may vary considerably (12). Rats of the nalorphine group showed great distribution on both sides of the control results. A partial factor may be the varying effect of nalorphine on individuals. Our material was not great enough to consider the small differences obtained significant.

2) It may also be considered that the results of Lande and Bentley were to some extent unspecific, being obtained with such great concentrations of drugs (3 mM/l) that it is not possible to obtain them *in vivo* without stopping respiration. Neither are the concentrations of the drugs observed in the brain tissue exact because the activity or the inactivity of morphine in animal tissues is difficult to estimate. It may be that changes *in vivo* remain within the limits of the changes in »bound« ACh. These changes remained minimal in our experiments too.

3) Morphine when given *in vivo* could liberate some substance having an antagonistic effect upon it. If we were to presume, too that the substance were produced in some other organ and then transported to the brain we could easily explain the differences *in vivo* and *in vitro*. Substance P is antagonistic to morphine in some cases (19) but it is not known whether it can be a physiological antagonist.

4) Morphine and nalorphine when given *in vivo* do not alter ChE activity as much as was found to be the case *in vitro*. The inhibition *in vitro* has been clearly noted using the purified enzyme preparate (7). The causes of the differences *in vitro* and *in vivo* have been discussed earlier. ChE is an enzyme with such great reserves that it is very difficult to determine changes in its activity, especially when this becomes increased.

SUMMARY

The action of nalorphine on morphine induced convulsions and the action of lethal doses of nalorphine were studied using rabbits as test animals. The same drugs were administrated to rats intraperitoneally. After decapitation the liberating brain acetylcholine (ACh) and the cholinesterase (ChE) activity in the brain and spinal cord were determined.

1) Nalorphine diminishes morphine convulsions of the rabbit to some extent. On the other hand if the convulsions are extremely strong nalorphine enhances them and quickens the death of the animal.

2) Great doses of nalorphine provoke convulsions in the rabbit.

3) These drugs have no effect on ChE activity. Great doses of morphine tend to inhibit the liberation of brain ACh to some extent but the differences obtained are not significant.

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FROM THE INSTITUTE OF FORENSIC MEDICINE AND THE DEPARTMENT OF
SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

VARIATIONS IN THE TISSUE COMPONENTS OF THE TUBERCULOUS LYMPH NODE

STUDIES ON GUINEA PIGS USING THE LINE SAMPLING METHOD

Preliminary Report

by

MARTTI KOKKONEN and HILKKA HEIKKILÄ

(Received for publication January 10, 1959)

The authors have studied the changes in the tissue components of the tuberculous lymph node in the guinea pig during ten weeks following inoculation. Tuberculous lymph node infection has been studied previously, but by qualitative or semiquantitative methods. The present authors aimed at a quantitative system and developed a method in which line sampling (10) was used to express as percentages the tissue components of the tuberculous lymph node. This study is of preliminary nature and is of essential importance from the point of view of our continued studies of the subject. The work also included experiments with a view to determining the effect of streptomycin on the tissue components of the tuberculous lymph node.

MATERIAL AND METHODS

Twenty female guinea pigs weighing on an average 400 gm. were used for the experiments. The guinea pigs were inoculated with a virulent bacterial strain pathogenic in man (H_37Rv ; 0.1 mg. per animal, wet weight);

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the injections were given subcutaneously into the left inguinal fold of the guinea pig. The 20 guinea pigs were divided into two groups, one control group infected with tuberculosis, and one similar group treated with streptomycin. Treatment was started on the 21st day after infection. An intramuscular dose of 10 mg. per animal every second day was considered the most suitable one.

Two guinea pigs in each group were killed at intervals of two weeks. The last group, surviving for 10 weeks after inoculation, thus received streptomycin 50 days. The development of streptomycinresistance was feared, and a sample was sent for resistance assay at the 8-week point; the result showed full sensitivity to streptomycin. The tuberculous lymph nodes from the left inguinal fold were removed from each animal for histopathological examination. In addition, the endocrine glands (pituitary, thyroid, adrenals, gonads) were weighed, and the liver, spleen and kidneys preserved for histopathological examination. Histological sections were cut on each side of the 50% line from the lymph nodes of the left inguinal fold. The sections were analysed by the line sampling method using a microp projector described and developed by Uotila and Kannas 1952 (10).

RESULTS

The variations in the typical tissue components of the tuberculous lymph node are shown in tables 1, 2 and 3, and in the graphic representation (fig. 1). The results are expressed as percentages of the length of the sampling line. The variations in the tissue components of one lymph node were first studied. These variations

TABLE 1
TISSUE COMPONENTS ANALYSED AT 2-WEEK INTERVALS (EXPRESSED AS PERCENTAGES)

Control group

The animals of this group are indicated by capitals (A, B)

Tissue	1A	1B	2A	2B	3A	3B	4A	4B	5A	5B
Connective										
tissue	11.4	8.7	2.9	4.2	6.5	18.4	7.5	11.0	12.0	18.0
Lymphatic										
tissue	32.6	35.2	21.3	32.8	39.3	71.5	66.4	56.6	22.1	32.0
Epithelioid										
cells	34.8	24.6	42.0	45.8	29.5	5.1	6.1	8.6	17.2	3.0
Giant cells	—	—	0.1	0.1	—	—	—	—	—	—
Inflammatory										
cells	21.2	31.5	33.7	8.6	12.3	4.1	4.3	0.2	0.5	3.0
Caseous										
necrosis	—	—	—	8.5	12.4	0.9	15.7	23.6	47.9	44.0
	2		4		6		8		10 weeks	

Streptomycin group

The animals of this group are indicated by small letters (a, b)

Tissue	1a	1b	2a	2b	3a	3b	4a	4b	5a	5b
Connective tissue	6.0	9.8	5.1	5.8	7.6	8.8	12.9	14.3	10.7	7.6
Lymphatic tissue	13.2	34.6	30.0	40.8	38.5	57.8	62.9	59.1	35.9	23.4
Epitheloid cells	26.5	39.3	57.4	26.4	23.0	16.5	23.4	18.1	21.9	13.2
Giant cells	—	—	0.2	—	—	—	—	—	—	—
Inflammatory cells	54.3	16.3	2.1	23.7	4.2	3.4	0.8	0.9	2.1	0.6
Caseous necrosis	—	—	5.2	3.3	26.7	13.5	—	7.6	24.9	55.2
			2	4	6	8			10 weeks	

The means of the tissue components (from table 1) analysed at 2-week intervals.

TABLE 2
SUMMARY OF PRECEDING TABLE (EXPRESSED AS PERCENTAGES)

Control group

Tissue	1A+1B	2A+2B	3A+3B	4A+4B	5A+5B
Connective tissue ..	9.3	3.6	11.1	9.1	14.7
Lymphatic tissue ..	34.3	27.3	52.0	62.1	27.6
Epitheloid cells	28.9	44.9	19.6	7.7	10.5
Giant cells	—	0.1	—	—	—
Inflammatory cells..	27.5	19.7	9.2	2.2	1.8
Caseous necrosis ..	—	4.4	8.1	18.9	45.4
	2	4	6	8	10 weeks

Streptomycin group

Tissue	1a+1b	2a+2b	3a+3b	4a+4b	5a+5b
Connective tissue ..	7.6	5.2	7.7	11.6	8.3
Lymphatic tissue ..	23.3	35.4	48.0	62.2	28.9
Epitheloid cells	33.1	41.9	19.2	21.2	16.6
Giant cells	—	0.1	—	—	—
Inflammatory cells..	36.0	12.8	4.1	1.0	1.0
Caseous necrosis ..	—	4.5	21.0	4.0	45.2
	2	4	6	8	10 weeks

TABLE 3
TISSUE COMPONENTS OF THE INGUINAL LYMPH NODES OF HEALTHY GUINEA PIGS (EXPRESSED AS PERCENTAGES)

Tissue	I	II	I+II
Connective tissue ..	10.8	6.1	8.45
Lymphatic tissue	89.2	93.9	91.55
Epithelioid cells ..	—	—	The means
Giant cells	—	—	—
Inflammatory cells	—	—	—
Caseous necrosis ..	—	—	—

were calculated mathematically, and the method was found to be accurate. In the present study five sections were taken from each lymph node, and the means for the tissue components of these five sections were expressed as percentages. The sections were derived from both sides of the 50% section (10). The standard error of the percentage on five sampling lines was calculated to be 1.7% for connective tissue, 2.9% for lymphatic tissue, 0.7% for epithelioid cells, 0.6% for inflammatory cells, and 2.7% for caseous necrosis.

Connective Tissue. — The amount of connective tissue, expressed as a percentage, shows a decrease at the 4-week point in both experimental groups. This is probably due to the fact that the size of the lymph node increases and the infection is in the initial phase, the amount of connective tissue being the same as before. As infection progresses, the connective tissue seems to increase gradually, which is shown graphically in figure 1. The two groups do not seem to differ appreciably.

Lymphatic Tissue. — Tissue in which lymphocytes predominated was regarded as lymphatic tissue. It evidently decreases during the first few weeks as compared with the normal level. After this initial decrease the percentage of this tissue increases up to the 8-week point, but then again seems to decrease abruptly, probably due to an increase of necrotic tissue. No significant difference is observed between the groups of animals studied. This is clearly apparent from the graphic representation.

Epithelioid Cells. — The epithelioid cell reaction in the lymph node sections is already intense at the 2-week point and it increases up to the 4-week point. Thereafter the epithelioid tissue begins to

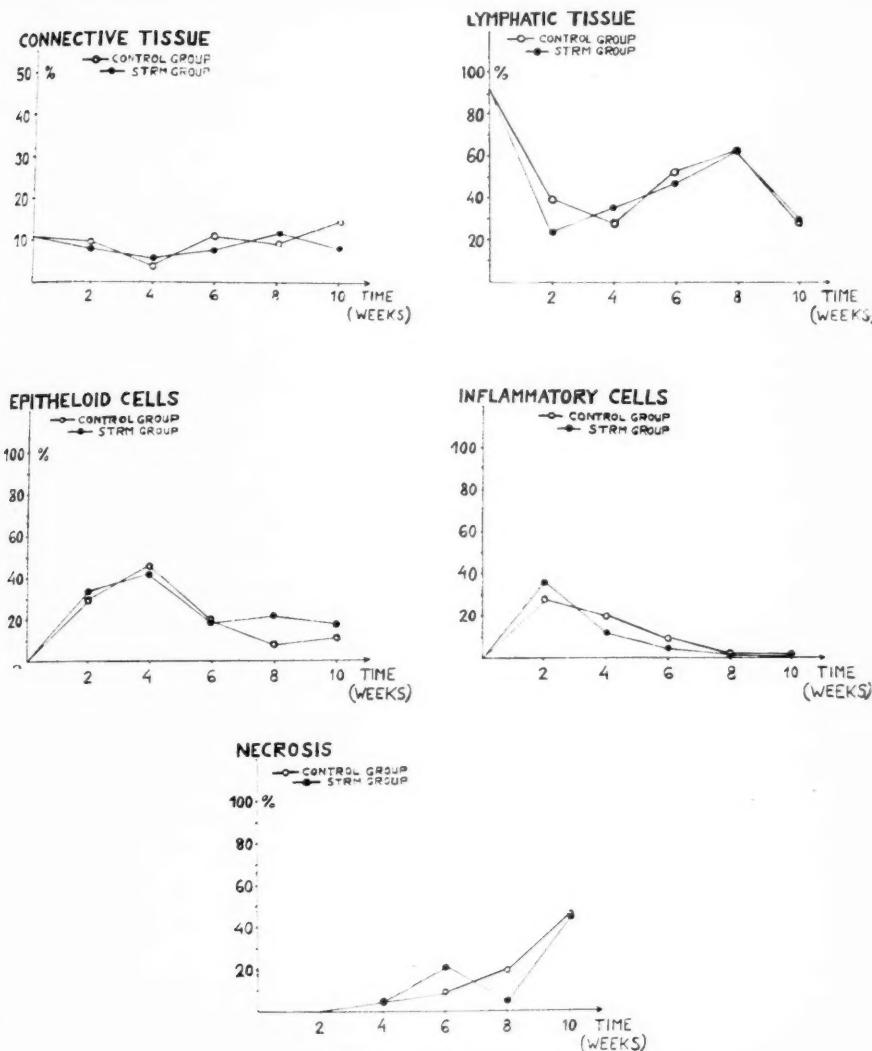


Fig. 1. — Graphic representation of the extent of variation of the different tissue components.

diminish steadily with progression of the disease. A large part of the epitheloid tissue seems to become necrotic, especially in the central portions of the node. A slight difference is noted at the 8-week and 10-week points between the streptomycin-treated group and the control group. This seems to support the results of some

other investigators. Flory *et al.* (1948) and Huebschman (1949) (cited by Canetti 1955) (2) observed that streptomycin causes an increase in epitheloid tissue either by increasing the counter-reaction of the tissue or by exerting a chemical mitogenic effect.

Giant Cells. — These seem to appear between the 2-week and the 4-week point. Giant cells are the most numerous at the 4-week point. These cells are so small in number as not to influence the tissue components expressed as percentages. The giant cells are easily recognizable but occur far apart in the tissue. Those seen in lymph node tissue were typical Langhans cells.

Inflammatory Cells. — Tissue in which polymorphonuclear cells predominated was regarded as inflammatory tissue. Inflammatory cells occur in the tuberculous lymph node of the guinea pig at the 2-week point, and in fairly great numbers. This is probably due to the secondary infection, because it raveled out an open tuberculosis in the left inguinal fold of the guinea pigs. Inflammatory cells seem to decrease with an increase in the lymphatic tissue and caseous necrosis in the later stages of infection. No distinct difference is noted between the control group and the streptomycin-treated group.

Caseous Necrosis. — Lymph node necrosis seems to appear between the 2-week and the 4-week point almost simultaneously with giant cell reaction. The necrosis consists of typical caseous necrosis, cell destruction. Necrosis increases in both groups until the end of the experiment or the 10-week point, which is clearly evident from the graphic representation.

DISCUSSION

It was the purpose of this work to study more closely the variations occurring in the tissue components of the tuberculous lymph node with progression of infection, a quantitative system being aimed at. Uotila and Kannas (1952) (10) used the line sampling method for determining the tissue components of the thyroid gland.

The typical tissues of the tuberculous lymph node were selected as the components to be studied: connective tissue, lymphatic tissue, epitheloid cells, giant cells, inflammatory cells (polymorphonuclear cells), and caseous necrosis. Several investigators have dealt with the variations in the tissue components of the

lymph node from the qualitative or semiquantitative point of view, for example the Japanese Nagai (1955) (6). The objective of the present investigation was to obtain typical quantitative curves illustrating the changes in the tissue components with progression of the disease.

The material is small, but in our opinion adequate for demonstrating that the method can be used in quantitative analysis of the tuberculous lymph node. The effect of streptomycin on lymph node tuberculosis was also studied.

Figure 1 shows clearly the interrelationships of the tissue components, expressed as percentages, at various stages of the disease. The connective tissue is found to increase gradually as the disease progresses. The fibroblast reaction is prominent at the repair phase. The curves of the control group and the streptomycin group agree entirely.

Compared with the normal, the lymphatic tissue, expressed as a percentage, decreases greatly during the first two weeks. This is due to migration of mononucleate and polymorphonuclear leukocytes. The lymphatic tissue increases from the 2-week point up to the 8-week point, but then this tissue diminishes, evidently as a result of necrosis. Epitheloid cells appear about 10 days after inoculation (6). Their number is greatest at the 4-week point. At this point tissue necrosis appears, which then increases at first owing to the lymphocytes and leukocytes undergoing necrosis, but also at the expense of epitheloid cells and in later stages at the expense of lymphatic tissue in great masses. The epitheloid cells occupy a central site in the tubercle and more easily undergo necrosis than the giant cells.

Giant cells appear in the lymph node between the 2-week and the 4-week point and they are typical Langhans cells. The giant cells, however, occur far apart and may also be found in the middle of a necrotic area.

The material is too small to permit conclusions regarding the effects of streptomycin. It is possible that streptomycin causes a greater epitheloid cell reaction than is seen in the control group.

SUMMARY

The authors have studied the variations occurring with progressing infection in the tissue components of the tuberculous lymph node of the guinea pig during 10 weeks after inoculation. The line sampling method (Uotila and Kannas 1952) (10) was used. A method was developed for analysis of the tissue components of lymph nodes. It can be concluded from the study that the line sampling method is suited for determination of the tissue components of the tuberculous lymph node. The effect of streptomycin on tuberculosis of the lymph nodes was also dealt with. The material is not large enough for conclusions to be drawn regarding streptomycin. It is possible that the epitheloid cell reaction induced by streptomycin is greater than that seen in the control group.

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FROM THE INSTITUTE OF FORENSIC MEDICINE (CHEMICAL DEPARTMENT),
UNIVERSITY OF HELSINKI

USE OF ACETONE IN THE ISOLATION OF ORGANIC POISONS FROM BIOLOGICAL MATERIAL

by

ANTTI R. ALHA and RAIMO O. LINDFORS

(Received for publication January 22, 1959)

In 1934 Chéramy and Lobo (3, 4) published a modification of the Stas-Otto method, in which, instead of ethanol, acetone was used as solvent for the isolation of barbiturates from biological material. By use of this method the compounds in question may be isolated more rapidly, in a purer form and with better yield than by the Stas-Otto method (7, 8). We have used this method in our laboratory for the detection of barbiturates with good results. This gave rise to the thought that acetone may be suitable for use as common solvent in the isolation of organic »poisons» other than barbiturates from biological material, too. In preliminary parallel experiments using methanol, ethanol and acetone as solvents the last one was found to be most suitable.

A more quantitative methodical study was then made with substances added to specimens from human organs. In the final extraction comparison was made between the shaking and perforation methods.

The following »mixed organs» were employed: Biological material from cadavers (in which poisonous substances could not be detected): One-fourth of the total amount was intestine with contents (no stomach contents, which usually are examined separately), one-third of it liver and the rest of it kidney, spleen and brain. To these »mixed organs» were added 50 mg of each of five selected type substances per 100 g of material, *i.e.* carbromal, phenobarbital,

amidone, dionine and morphine (the three last mentioned as hydrochlorides).

Conforming with the usual toxicological usage, final extraction was made into four consecutive fractions: Group I: Substances dissolving in ether from acid solution (carbromal and phenobarbital); Group II: Substances dissolving in chloroform from acid solution (amidone); Group III: Substances dissolving in ether from NaOH alkaline solution (dionine), and Group IV: Substances dissolving into a mixture of chloroform and methanol (10 per cent methanol) from NaHCO₃ alkaline solution (morphine).

PROCEDURE

1. *Acetone Method.* — The ground »mixed organs» (100 g) are made acid (pH ca 4) with tartaric acid. Then 200 ml of 70 per cent acetone (purum Merck) is added. The mixture is heated in a water bath (60°C) with a reflux condenser for ½ hr. It is then kept at room temperature for a few hours, preferably overnight. It is filtered and the precipitate is washed with acetone (70 per cent).

— The acetone is removed from the filtrate in vacuum by distillation (60°C), which is continued until the flask contains ca 50 ml of liquid. After cooling the distillation residue is rinsed with water into a beaker.

— The distillation residue is made slightly but clearly alkaline with NH₄OH and then acid once more with tartaric acid. The solution obtained (»fatty mass») is filtered and the precipitate is washed with water.

— Ca 400 ml of acetone is gradually added to the filtrate, which is then kept at room temperature until the precipitate has descended to the bottom. It is filtered and the precipitate is washed with acetone.

— The acetone is removed from the filtrate in vacuum, as before, and distillation is continued until the flask contains 10—15 ml of liquid. When cool, another acetone precipitation is carried out with 400 ml of acetone. After descending of the precipitate filtration is carried out and the precipitate is washed with acetone.

— The acetone is removed from the filtrate as before, then 50 ml of hot water is added. After cooling the solution is filtered (»aqueous solution»).

— During the procedure all the precipitates are saved for possible additional examinations.

2. *Simplified Acetone Method.* — For extraction of the ground »mixed organs», which have been rendered acid, a corresponding amount of undiluted acetone is used. Heating and filtration are carried out as above.

— To the filtrate is added ca 25 g of anhydrous sodium sulphate to saturation. The mixture is shaken mechanically for ½ hr, then allowed to stand at room temperature for some hours, after which it is filtered and the precipitate is washed with acetone.

— The acetone is removed from the filtrate by distillation.

— The distillation residue (»fatty mass«) is rinsed from the flask with hot water to make 50 ml. After cooling it is filtered (»aqueous solution«).

3. *Extraction of »Aqueous Solution«.* — *Shaking.* — It is carried out in each group with 3×25 ml of solvent. The combined extracts are dried with anh. sodium sulphate. Before extraction of Group IV the aqueous solution is heated in a boiling water bath for $\frac{1}{2}$ hr after making acid with HCl, after which the solution is made alkaline with NaHCO₃. After evaporation of the solvents the residue in Groups III and IV is converted to hydrochlorides by evaporation to dryness with dil. HCl.

— *Perforation.* — This is carried out with glass perforators of the common type accomodating 100 ml of aqueous solution. In each group 75 ml of solvent is used. The perforation times are 2 hours for perforation with ether in Groups I and III and 6 hours for chloroform and chloroform-methanol perforation in Groups II and IV. The extracts obtained are treated then as in the shaking method.

4. *Purifications.* — In Group I the purification of the crude product is carried out by dissolving in ether and passing through an animal charcoal-MgO column (1, 6). Purification in Group II is made by the same method, using chloroform as solvent. The crude products in Groups III and IV are not purified.

5. *Identifications.* — After weighing, the purified products in Groups I and II and the crude products in Groups III and IV are identified. The bromine of carbromal is demonstrated by the fluoresceine test (5). Phenobarbital is demonstrated by a modified Zwicker test (13) and by paper chromatography (9, 10). Amidone is identified by the cobalt thiocyanate reaction (2, 12) and by paper chromatography (11), dionine and morphine with Fröhde's reagent and by paper chromatography (11), too.

EXPERIMENTAL

Preparatory experiments were first made, using the simplified acetone method and extraction by shaking.

The used type substances were added 1) into the »mixed organs«, 2) into the »fatty mass«, 3) into the »aqueous solution«. It was noted that the loss of yield distinctly increased in every successive phase of procedure.

Further it was observed that the type substances were not sufficiently separated from each other into different groups. This prompted the use of the extraction by perforation.

The actual experiments were then made with simplified acetone method using parallel extractions by shaking and by perforation. As control served 100 g of »mixed organs« with no added substances. The results of weighing of one experimental series are shown in

TABLE 1

RESULTS OF WEIGHING, IN MG. SIMPLIFIED ACETONE METHOD. PARALLEL EXTRACTION BY SHAKING AND PERFORATION

Group	With Added Substances		Controls	
	Shaking	Perforation	Shaking	Perforation
I Crude Product	99.8	139.4	12.1	15.8
Purified	90.2	100.4	1.4	1.5
II Crude Product	36.1	46.2	6.1	9.6
Purified	31.6	40.0	1.5	4.0
III Crude Product	32.8	41.6	2.8	7.2
IV Crude Product	10.4	20.0	1.2	1.6

Table 1. For Group I the results of weighing are, of course, the combined yields of carbromal and phenobarbital.

In the identification the control series not only gave negative reactions but also no spots in paper chromatography. In the series with added substances it was observed that the separation of the type substances into the different groups was possible when using perforation. On the other hand — as was already referred to above — the shaking method yielded some carbromal and phenobarbital in addition to amidone in Group II, amidone in addition to dionine in Group III, and dionine in addition to morphine in Group IV. Thus the weighing yields obtained merely by shaking do not indicate the amount of type substances separated in the «pure» state.

Further, using perforation for extraction a corresponding comparison between the acetone method and the simplified acetone method was performed. The weighing yields obtained in a series of this kind are shown in Table 2.

TABLE 2

RESULTS OF WEIGHING, IN MG. PARALLEL ISOLATION BY THE ACETONE AND THE SIMPLIFIED ACETONE METHODS

Group	With Added Substances		Controls	
	Acetone	Simplified	Acetone	Simplified
I Crude Product	172.9	122.3	80.2	17.0
Purified	75.7	94.7	5.7	2.9
II Crude Product	75.5	44.2	17.9	5.6
Purified	62.1	39.6	8.2	3.7
III Crude Product	28.0	38.2	3.7	6.4
IV Crude Product	15.0	25.6	2.2	2.8

The conclusions may be drawn from Table 2 that the amount of impurities extracted in the different groups by the acetone method was markedly greater in Groups I and II and somewhat smaller in Groups III and IV than when the simplified acetone method was used. Furthermore it may be concluded that the losses when using simplified acetone method are — owing to fewer number of phases in the procedure — smaller than those with acetone method.

The weighed yields according to Tables 1 and 2 obtained by the perforation may therefore be regarded as indicative of the yields of the added substances. The relative yield percentages, after taking into consideration the results of weighing in the control group, are as follows:

		Isolation Method
	Acetone	Simplified
Group I	Carbromal + phenobarbital ca 70%	ca 92—99%
II	Amidone · HCl (108%?)	72%
III	Dionine · HCl 49%	64—68%
IV	Morphine · HCl 26%	37—46%

It will be observed according to Table 1 that in Group I even the yield obtained by the shaking method (ca 89%) of carbromal + phenobarbital is sufficient to permit the use of shaking alone in practical forensic chemical analysis.

Additional Experiment. — To obtain an indication of the serviceability of the above presented acetone method in examinations on a wider scale we carried out qualitative experiments by adding to the same batch of 100 g of »mixed organs» 10 mg of each of the following substances: 3-(2-methylphenoxy)-propane-1,2-diol (Myanecin), phenylchinoline carbonic acid (Cincophen), caffeine, benadryl, chloropromazine, quinine and strychnine. Isolation was carried out by the simplified acetone method and group extraction by perforation. All the added substances were very readily identified.

INVESTIGATION OF FATAL CASES

During the period February 1957—July 1958 we investigated in this institute samples of organs in 381 fatal cases by the acetone method. A negative result for organic »poisons» was obtained in 144 of these cases. In the 237 cases with positive results the investigation was performed from »mixed organs» in 221 cases, from the urine in 65 cases, from stomach contents in 29 cases, and from

the blood and from other organs in some cases. Table 3 gives data on the substances identified.

Identification was carried out chiefly by using m.p. and mix. m.p. det., spot-tests, paper chromatography, spectrophotometry and infrared spectroscopy.

TABLE 3

DISTRIBUTION OF IDENTIFIED SUBSTANCES IN THE PERIOD FEBRUARY 1957—JULY 1958. NUMBER OF IDENTIFICATIONS

Bromine containing substance	58
Acetanilid	1
Acetophenetidin	2
Bemegride (Megimide)	1
Ethinamate (Valamin).....	1
Meprobamate (Miltown)	2
Mesantoin	1
Sedormid	1
Unidentified substance in Group I (Doriden?)	5
Parathion	3
p-Nitrophenol	11
Phenolphthalein	2
Salicylate	48
Barbiturates	234
Purine deriv.	10
Papaverine	2
Amidone	1
Antipyrine	15
Aminopyrine	19
Benadryl.....	25
Promazine deriv.	10
Unidentified substance in Groups II—III (Cliradon?) ..	1
Chloroquine	2
Nupercaine	1
Tetracaine	1
Nicotine	2
Quinidine	1
Quinine	7
Strychnine or Brucine.....	3
Atropine	1
Scopolamine	1
Unidentified mydriatic substance in Group III	4
Codeine	3
Unidentified substance in Group III (Heroin? Dionine? other opiatics?)	5
Morphine	6

COMMENTS

In methodical investigations using the acetone method, batches of 10–50 mg of selected neutral, acid and alkaline organic substances added to »mixed organs» were isolated. Furthermore, in numerous fatal cases of poisoning the use of this method gave consequent analysis findings from cadaverous materials.

In the practical application the importance of the conjugates possibly formed as products of detoxication was not studied. As is well known, certain alkaloids, for example, form conjugates, the decomposition of which requires treatment with strong acids, autoclaving etc.

A methodical investigation of relative quantity was performed with five selected type substances added to »mixed organs», which frequently are an adequate investigation object in practical forensic chemical analysis. Since, however, the composition of a mixture of organs may vary within a very wide range, the presented yield percentages have a relative value only. Individual organs, urine and blood, on the other hand, will probably give quantitative results of a greater and wider validity.

The procedure for protein precipitation may be regarded as adequate in many cases. However, if the stage of putrification of the material to be investigated is far advanced, there may be reason to perform more precipitations with acetone or to use combined acetone-ethanol precipitation, etc.

To determine the applicability of the method for the isolation of a new substance a separate preliminary investigation may be necessary.

Subjects requiring further study are the development of methods of additional separation and purification.

SUMMARY

1. An acetone method and its simplified modification are presented for use as a general procedure for the isolation of organic poisons from biological material.

2. Using this method, experimental investigations were made of the relative yields of carbromal + phenobarbital, amidone, dionine and morphine added to »mixed organs».

3. A qualitative investigation for isolation was carried out with seven substances selected at random, all of which were readily identifiable in the extracts.

4. Statistics are presented on the chemical investigation of cadaverous materials from 381 suspected fatal cases of poisoning. In 237 cases a positive identification of an organic compound was obtained. The number of identified compounds or types of compounds was 35 (given in tabulated form).

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FROM THE INSTITUTE OF FORENSIC MEDICINE (CHEMICAL DEPARTMENT),
UNIVERSITY OF HELSINKI

INFRARED SPECTROSCOPY IN FORENSIC CHEMICAL IDENTIFICATION

CHEMICAL INVESTIGATION OF 31 FATAL CASES OF SUSPECTED POISONING

by

ANTTI R. ALHA and VEIKKO TAMMINEN

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Infrared spectroscopy is the best and most rapid method for the identification of pure organic compounds when the spectra of these compounds are available.

Since 1957 the Institute of Forensic Medicine of the University of Helsinki has been equipped with a Perkin-Elmer M 13 infrared spectroscope with attached microscope M 85¹. As is well known, this equipment can be used for the examination of, for example, 0.5—1.5 mg solids. When the microscope is used the amount of substance needed is considerable smaller — in some cases a few micrograms will be sufficient (2).

The method, therefore, is applicable to the identification of the frequently minute amounts of pure substances available for forensic chemical analysis. We have recently reported an investigation of this kind (6), in which infrared spectroscopy of a few drops of liquid left by two deceased persons definitely revealed the cause of death (ethylene glycol).

A different situation prevails when the poison is to be detected from samples of organs of the deceased person. To obtain from

¹ This equipment was presented to the Institute by the Rockefeller Foundation.

material of this kind a sufficiently pure substance for identification it is necessary to carry out various, often complicated, separation and purification procedures, frequently at the considerable expense of the yield of toxic substance. It is, therefore, obvious that the separation and purification techniques used have a decisive influence over the identification of the substance.

In this paper we present the chemical investigation of body organs in 31 fatal cases, in which infrared spectroscopy was used for identification. In 5 of these cases the result of the investigation was negative.

Only the examinations leading to the infrared spectroscopic finding are described. Details of other identification techniques belonging generally to our routine analysis (such as spot tests, melting point determinations, and paper chromatographic and spectrophotometric examinations) are also omitted except where they are considered to have a bearing on the infrared spectroscopic investigation. Furthermore, since this is a qualitative investigation, the weights of certain separated products and the results of other possibly performed quantitative determinations are not stated in every case.

In all the cases the autopsy material sent for forensic chemical investigation consisted of the solid viscera, stomach, intestine with contents, and blood, in several cases urine, in many cases stomach contents, and in some cases drugs, bottles and other objects left by the deceased.

TECHNIQUES FOR SEPARATION AND PURIFICATION

I. Routine Methods

Separation

- A. Direct separation. Case 1.
- B. Separation with distillation. Material for distillation was made acid with tartaric acid.
 1. Direct steam distillation (150 ml). Cases 2, 3 and 7, and partly Case 28.
 2. Steam distillation after acetone procedure. To the material a four-fold amount of acetone was added gradually with continuous stirring, the mixture was kept in a mechanical shaking machine for $\frac{1}{2}$ hour and filtered. The acetone was then carefully removed by evaporation and the residue was steam distilled. Cases 4—6 and partly Case 24.
- C. Separation of nonvolatile compounds. Cases 8—23, 25—27, 29—31 and partly Cases 24 and 28. For isolation, acetone was used according to the working instruction developed in the Department of Forensic Chemistry (see this Journal, p 149). An exception from the instruction was made in Case 27 (morphine), in which the urine under examination, instead of using

tartaric acid, was made strongly acid with HCl and kept with a reflux condenser on the water-bath for one day.

The aqueous solutions obtained as the end product of acetone treatment were extracted routinely into four groups in the following order:

- Group I: Extraction of acid solution with ether;
- » II: » » » chloroform;
- » III: » NaOH-alkaline solution with ether;
- » IV: » NaHCO₃-alkaline solution with hot methanol-chloroform (1: 10).

An exception was Case 10, in which the extraction was first made from the acid aqueous solution with ether and then from NH₃-alkaline solution with chloroform. Extraction was carried out with shaking in all cases with the exception of Case 27, in which the object of examination, Group IV, was extracted by perforation.

The object of investigation in Case 10 was the chloroform extract, in Cases 15—18 Group III, in Case 27 Group IV, and in the remaining 18 cases Group I.

In all the cases, purification and/or additional separation procedure was carried out in Group I.

Purification and Additional Separation Procedures

1. *Water Purification*. — The Group I crude product was dissolved in 50—200 ml H₂O. The solution was heated in the water-bath for ½ hour and allowed to cool. Fatty and resinous impurities form droplets on the sides of the vessel or on the surface of the solution. After filtration of the solution the filtrate was shaken with ether. The ether was evaporated or an additional separation was carried out.

Additional separation (separation of acid and neutral substances). — Ether or its aliquot was shaken once with 5 ml of 0.1 N NaOH, then with water, 0.1 N H₂SO₄, and water. The evaporation residue of the ether contains the neutral compounds, Group I_n. The alkaline layer was made acid with dilute H₂SO₄ and shaken with ether, which was washed with water. The evaporation residue of this ether solution contains the acid compounds, Group I_{ac}.

2. *Treatment with Phosphate Buffer pH 7.2*. — The primary ether solution obtained in the shaking of Group I (for example 75 ml), the ether solution of the crude product, or its aliquot was shaken once with 30 ml of buffer. The ether solution was washed with water and evaporated to dryness, or additional separation was carried out as above. The latter gave Group I_n and Group I_{wac} (weakly acid compounds).

3. *Sublimation*. — This was carried out in the usual manner using Kofler's hot stage.

II. Special Separation Methods

- A. Ion exchange with Dowex 2. Cases 25—27.
- B. Chromatography on silica gel. Cases 28—29.

INFRARED TECHNIQUES

All the spectra were taken using NaCl prism. A, B and C techniques presented below give a »Double Beam» spectrum; D technique gives a »Single Beam» spectrum. In A and B techniques the liquid to be examined was saturated with solid NaCl.

- Technique A. — Spectra of liquid, 0.0264 mm NaCl cell.
- Technique B. — The substance was dissolved in CS₂, 0.103 mm NaCl cell; CS₂ in 0.102 mm NaCl cell.
- Technique C. — KBr disc technique. Vibrator acc. to v. Ardenne; Vacuum Press Tool acc. to Dr. Schiedt, type 8; Hydraulic Labor-Handpress, PW 20, Paul Weber, Stuttgart-Uhlbach; KBr (for Infrared Spectrography, Merck) was powdered and sieved (80 Din).
 - C 1: Ca 1.5 mg of substance in 300 mg KBr.
 - C 2: The substance dissolved in ethanol was added to powdered KBr and the solvent was evaporated.
 - C 3: KBr disc (ca 300 mg). Viscous substance was smeared on the surface of the disc.
 - C 4: KBr disc (ca 300 mg). The substances sublimated on a slide was dissolved in CCl₄ and the solution was dropped on the disc, simultaneously evaporating the solvent.
 - C 5: KBr disc (100—300 mg). The substance was sublimated on the disc.
- Technique D. — The spectrum of an isolated single crystal was taken in the infrared microscope on a KBr disc (100 mg).

The techniques used in each case are shown in Table 3.

The statements made below of the result of the infrared spectroscopic examination in each case (= Spectrum) is based on an identity with the spectrum of a known pure compound, unless otherwise stated. The comparison was made either against the spectra taken of pure substances chosen on the basis of the originally suspected course of events, on the basis of indications obtained from the results of other chemical identification methods of the substance under examination, or by examining the already available spectra of known compounds.

INVESTIGATION OF CASES

The suspected poisoning stated in the case reports is given for each case in Table 1.

TABLE 1
SUSPECTED POISONING ACCORDING TO CASE HISTORY

Case No.	D No.	Suspected
1	238/57	Trichloroethylene
2	567/58	Trichloroethylene
3	595/58	Parathion
4	294/58	Parathion
5	587/58	Parathion and DDT
6	132/58	Systox
7	631/58	? In autopsy an odd smell was noted: Camphor or organic phosphate insecticides? Pathologically arteriosclerosis gravis univ. was found
8	158/57	? Child of 3 months, sick with fever
9	237/57	? Found dead
10	305/57	Aminopyrine tablets accidentally. Child of 2 years
11	176/58	Sedormid
12	184/58	Hypnotics
13	418/58	Hypnotics
14	755/57	Phenobarbital tablets. In hospital given as antidote Malysol (Megimide)
15	196/58	? Found dead, close by drugs of various kinds, among others Nivaquine (M & B, Rec: Chloroquine sulphate 0.2)
16	402/58	Chloroquine tablets 1.5 g as anthelmintic. Child aged 4
17	42/58	Hypnotics, among others Pentobromital tablets (Star, Rec: Na-Pentobarb. 0.1, Bromural 0.3)
18	420/58	Quinidine tablets accidentally. Child aged 2
19	177/58	? Found dead, close by drugs of various kinds, among others Meprobamate, Bromural, Amytal and Camphor
20	187/58	Amytal. Pathologically cirrhosis hepatis was found
21	192/58	? Found dead.
22	253/58	? Found dead, close by Valamin tablets
23	775/57	Found dead, close by an empty Hydantal tablet box (Sandoz, Rec: Mesantoin 0.1, Phenobarb. 0.02)
24	242/58	Valamin tablets
25	91/58	100 Soneryl tablets (M & B, Rec: Butobarbitone 0.097)
26	115/58	Meprobamate, Phanodorm tablets
27	247/58	Found dead. A chemist; close by an empty Phenobarbital tablet box and powder covers with some white powder
28	632/58	Found dead, close by 6 empty Carbital capsule bottles (P & D, Rec: Pentobarb. 0.1, Carbromal 0.26) and 3 white large tablets
29	660/58	Pentobarbital tablets
30	483/58	Found dead, close by a bottle with some white powder
31	637/58	Amytal tablets, Carbital capsules

The cases are grouped as follows. The cases first described are those in which the infrared spectroscopic examination result was usually obtained by routine separation, purification and additional separation techniques and in which the examination result allowed one interpretation only. In the second category are the cases in which the final separation was made by special methods. In the last two cases the spectrum of a mixture was obtained.

I. Routinely Investigated Cases (Cases 1—24)

The materials investigated in these cases are stated in Table 2.

TABLE 2
INVESTIGATED MATERIAL IN CASES 1—24

Material	Case No.
Stomach contents only (15—100 g)	1, 3, 4, 5, 6, 10
»Mixed organs« only (200—400 g)	2, 8, 13, 14, 15, 16, 18, 19, 21
Mixture of »mixed organs« (100—200 g), urine (25—100 ml) and stomach contents (5—100 g)	9, 11, 12, 17, 20
Mixture of »mixed organs« (100 g) and urine (100 ml)	22
»Mixed organs« (150 g) and stomach contents (150 g) separately	7
Liver (300 g), urine (200 ml) and stomach contents (50 g) separately	23
Liver, stomach contents, blood and »mixed organs« separately	24

The composition of »mixed organs« will be seen in a previous paper (1, see this Journal, p. 149).

Infrared spectroscopy gave a negative result in Cases 7 and 19—22.

Case 1. — At the bottom of the stomach contents there was ca 1 ml of separated fluid. It was isolated in a separatory funnel, shaken with water, dried with CaCl_2 , and distilled under ordinary pressure. The distillate was a clear, colourless liquid, Spectrum = *Trichloroethylene*.

Case 2. — Direct steam distillation of the investigated material. The distillate was shaken with 0.8 ml of CS_2 , Spectrum = *Trichloroethylene*.

Case 3. — Direct steam distillation of the investigated material. The distillate was shaken with petrol ether, which was shaken with NH_4OH , then with H_2SO_4 , with water and with anhydrous Na_2SO_4 , filtered and

TABLE 3
INFRARED TECHNIQUE IN THE INVESTIGATION OF CASES. — SUBINDEX AFTER
THE CASE NUMBER REFERS TO THE SPECTRUM IN QUESTION

Technique	Case No.
A	1
B	2, 3, 7 _a , 7 _b
C 1	4 _b , 9, 10, 11, 12, 13, 14 _b , 23 _c , 25, 28 _d , 28 _{b₁} , 28 _{e₁} , 28 _{b₂} , 28 _{e₂} , 29 _{a₁} , 29 _{b₁} , 29 _{c₁} , 29 _{a₂} , 29 _{b₂} , 31 _{a₁} , 31 _{b₁} , 31 _{c₁} , 31 _{d₁} , 31 _{e₁} , 31 _{a₂} , 31 _{b₂} , 31 _{c₂} , 31 _{d₂} , 31 _{e₂}
C 2	4 _a , 5 _a , 5 _b , 15, 16, 17, 18, 19, 20, 21, 22, 24 _a , 24 _b , 24 _c , 24 _d , 24 _e , 26, 27, 28 _{e₁} , 28 _{d₁} , 28 _{d₂} , 29 _{c₂} , 30 _a , 30 _b , 30 _e
C 3	6
C 4	8
C 5	23 _a , 23 _b
D	14 _a

evaporated to dryness. The residue was a brownish, viscous liquid. This was dissolved in 0.5 ml of CS₂. Spectrum = *Parathion*.

Case 4. — Stomach contents (50 g) was steam distilled (250 ml) after acetone procedure. The distillate was treated with petrol ether, as in the preceding case. The evaporation residue of the petrol ether was ca 97 mg of a viscous, brownish liquid, Spectrum_a = *Parathion*. The remainder of the residue was hydrolysed with KOH in a closed vessel in the water-bath. The solution was shaken with petrol ether and the alkaline layer was made acid with H₂SO₄ and shaken with ether. As evaporation residue of the ether there remained ca 52 mg of a crystalline substance, Spectrum_b = *Paranitrophenol*.

Case 5. — The investigated material was steam distilled after acetone procedure. The distillate was treated with petrol ether as in Case 3. The evaporation residue of the petrol ether was a viscous substance, Spectrum_a = *Parathion and impurities*. The residue of the steam distillation was shaken with petrol ether as above. Its evaporation residue gave with K₂Cr₂O₇ (3) a red colour (DDT) but Spectrum_b did not definitely demonstrate DDT (insufficient amount of substance).

Case 6. — The investigation material was steam distilled after acetone procedure, followed by shaking with petrol ether as in Case 3. The petrol ether was further shaken with Na₂CO₃ solution and water. The evaporation residue of the petrol ether was a viscous substance, Spectrum = *Systox and impurities*.

Case 7. — The investigation materials were treated by direct steam distillation (150 ml from the stomach contents and 500 ml from »mixed organs«). The distillate from the stomach contents had a weak odour resembling camphor. The distillates were treated as in Case 2. Spectrum_a and Spectrum_b from both materials: *No absorptions*.

In Cases 8—23 the separation of nonvolatile compounds was made with acetone (1).

Case 8. — The Group I crude product gave a positive resorcine test (phenacetin). Sublimation of the crude product yielded a crystalline substance, Spectrum = *Phenacetin*.

Case 9. — The Group I crude product weighed 510 mg. It gave a positive bromine test (eosine reaction). The crude product was slightly soluble in ether, and purification was therefore made by decanting several times with a few ml of ether. The residue was a crystalline substance, m. p. 134.5—135°C, Spectrum = *Phenacetin*.

Case 10. — The CHCl_3 -soluble residue (see p. 159) yielded on sublimation a crystalline substance, Spectrum = *Aminopyrine*.

Case 11. — Buffer treatment of Group I. After the evaporation of the ether 175 mg of a crystalline substance was obtained, m. p. 190°C, Spectrum = *Sedormid*.

Case 12. — Buffer treatment of Group I and additional separation. Group I_n consisted of 49 mg of a crystalline substance, which gave a positive bromine test, m. p. 137—145°C, Spectrum = *Bromural*. — Group I_{wac} weighed 125 mg and was found to contain (paper chromatography) (5) phenobarbital and ethylpentylbarbituric acid (amytal or nembutal).

Case 13. — Buffer treatment of Group I. The evaporation residue of the ether was 34 mg of a crystalline substance, bromine test positive, Spectrum = *Bromural*. — From the urine (30 ml) was also separated 5 mg of viscous substance which gave a positive bromine test. Additionally, methanol was found in the organs and the formic acid content of the urine was 25 mg/100 ml.

Case 14. — Water purification of Group I crude product and additional separation into Groups I_n and I_{ac} . In Group I_n a thin platelike crystal was isolated, 1 mm in diam., Spectrum_a = *Megimide*. M. p. of the crystal was 125—128°C. Group I_{ac} was a crystalline substance, m. p. 170°C; Spectrum_b = *Phenobarbital*.

Case 15. — Group III was investigated, Spectrum = *Chloroquine as hydrochloride*.

Case 16. — Investigation was carried out from Group III, which weighed 26 mg, Spectrum = *Chloroquine as hydrochloride*. — From the urine (25 ml) was separated 7 mg of Group III which gave a positive chloroquine reaction with cobaltothiocyanate (8), with $\text{K}_2\text{Cr}_2\text{O}_7$ — H_2SO_4 and by paper chromatography (7).

Case 17. — Group III was investigated, Spectrum = *Quinine as hydrochloride*. — Group I gave a positive bromine test; barbiturates could not be found.

Case 18. — Investigation was carried out from Group III, of which 15 mg was separated, Spectrum = *Quinidine as hydrochloride*. Fig. 1 shows this spectrum and the spectra of quinine and quinidine, both as hydrochlorides. — From the urine (15 ml) was separated 4 mg of Group III,

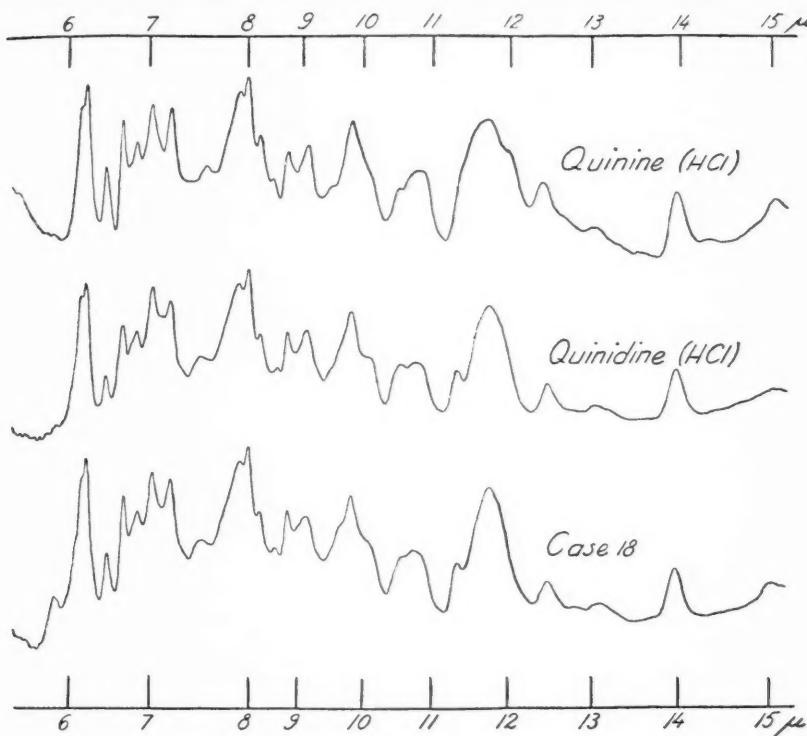


Fig. 1.

which gave the quinine reactions and paper chromatographically was identified as quinidine (and quinine).

Case 19. — Buffer treatment of Group I. The evaporation residue of the ether gave a doubtful bromine test. An additional separation was made from the residue. Group I_n: 14 mg of viscous substance, Spectrum: *Very low bands, no definite statistical hetero-atom groups.*

Case 20. — Buffer treatment of Group I. The evaporation residue of the ether was 53 mg. No barbiturates. Additional separation was made from one-half of the residue. Group I_n was 15 mg of viscous substance, Spectrum: *Negative* as in Case 19.

Case 21. — Buffer treatment of Group I. Yield: 60 mg, no barbiturates, bromine test doubtful. Additional separation and sublimation of Group I_n, Spectrum: *Negative* as in Case 19.

Case 22. — Buffer treatment of Group I. Yield: 57 mg, no barbiturates, bromine test negative. Additional separation from the remainder of the residue. Group I_n: 4 mg of viscous residue, Spectrum: *Negative* as in Case 19.

Case 23. — Groups I were separated from the investigated materials. Crude products were «water purified» and separated further

to Groups I_n and I_{ac}. Phenobarbital was found in all Groups I_{ac} (paper chromatography). Group I_n from stomach contents was not investigated further; other I_n Groups weighed as follows: I_n from liver 14 mg (a) and from urine 16 mg (b) of crystalline substances. Spectrum_a and Spectrum_b = *Mesantoin*. On separation of the stomach contents, the »fatty mass» obtained after distillation of the acetone and filtration was found to be filled with crystals. A separate examination was then made as follows: The »fatty mass» was dissolved in CHCl₃, filtered, the filtrate evaporated to dryness and washed by decanting with petrol ether. It was then dissolved in CHCl₃, shaken with HCl, NaOH, and water, dried, and purified with bone charcoal. The residue (c) after chloroform evaporation was 1684 mg of a white crystalline substance, m.p. 137—140°C, mixture melting point with mesantoin: No depression, Spectrum_c = *Mesantoin*.

Case 24. — Investigations were first made of a) 25 g of stomach contents, b) 195 g of liver, and c) 92 ml of blood. Steam distillation was carried out after acetone procedure as in Case 4, but the aqueous solutions obtained by filtration and removal of acetone by distillation were neutralised before distillation with dil. NaOH. The distillates (250—1000 ml) were shaken with ether. The evaporation residues of the ether were viscous, Spectrum_a = *Valamin*, but Spectrum_b and Spectrum_c: Negative as in Case 19. Investigations was then made of d) 100 g of stomach contents and e) 200 g of »mixed organs», using acetone for separation of »nonvolatile» compounds. Groups I were separated, and their buffer treatment and additional separation yielded Groups I_n: from stomach contents 308 mg and from »mixed organs» 24 mg. Both gave a positive bromine test. The residue of each Group I_n was steam distilled (500 ml). The distillates were shaken with ether. The evaporation residues of the ether gave a negative bromine test. The residue from stomach contents weighed 26 mg, Spectrum_d = *Valamin*, but that from »mixed organs» had a Spectrum_e: Negative as in Case 19.

II. Cases with Special Methods for Final Separation (Cases 25—29)

In these cases acetone was used for the routine separation of nonvolatile crude products.

A. Ion Exchange with Dowex 2

Case 25. — Buffer treatment and additional separation of Group I crude product (from 200 g of »mixed organs»). Group I_{wac} was ion exchanged through Dowex 2 (10 cm column, 5 ml resin, capacity 15 meq., OH⁻) with methanol as influent (3 × 10 ml). The column was washed with 3 × 10 ml petrol ether and eluted with 3 × 10 ml 0.5 N formic acid in methanol. The evaporation residue of the eluate was a crystalline substance, Spectrum = *Butobarbitone*.

Case 26. — Group I crude product (from a mixture of 200 g of »mixed organs», 25 ml of urine and 10 g of stomach contents) was ion exchanged

as in the preceding case. The evaporation residue of the influent was 15 mg of viscous substance, Spectrum = *Meprobamate*. — The evaporation residue of the eluate of formic acid in methanol weighed 11 mg and phanodorm was found in it (paper chromatography). According to quantitative UV spectrophotometric investigation by the method of Goldbaum (4) the total quantity contained 7.3 mg of phanodorm.

Case 27. — Powder covers contained a minute amount of white powder in which morphine was detected. — One-half of the Group IV HCl-treated evaporation residue (from 20 ml of urine) was taken in water, made NaHCO_3 -alkaline and perforated with CHCl_3 , after which the evaporation residue of the chloroform weighed 6 mg. This was ion exchanged as in Case 25. The evaporation residue of the formic acid-methanol eluate weighed ca 1 mg, Spectrum = *Morphine as formiate*. — Further, in 12 ml of urine, of which a preliminary examination was made, morphine and phenobarbital were found (paper chromatography), and in a mixture of 100 g of »mixed organs», 20 ml of urine and 50 g of stomach contents there was abundant phenobarbital, but morphine could not be found!

B. Chromatography on Silica Gel

Case 28. — First a) 100 g of liver was directly steam distilled (500 ml). The distillate was shaken with ether, the evaporation residue of which was »water purified». The evaporation residue of the ether was 4.5 mg of crystalline substance, bromine test positive, Spectrum_a = *Carbromal*. Investigation was then made of b) 200 g of »mixed organs», c) 50 g of stomach contents, and d) 37 ml of urine, using acetone for separation of »nonvolatile» compounds. The Groups I obtained were treated with buffer and the evaporation residues of the ether were »water purified». The following amounts of viscous residues were obtained: b) 419 mg, c) 274 mg, and d) 10 mg. Examination of the »fatty mass» obtained from the stomach contents after acetone treatment revealed abundant crystals as in Case 23. This was treated separately as follows: The »fatty mass» was dissolved in 500 ml of boiling water, allowed to cool to 30—35°C and filtered. The filtrate was shaken with ether, which was treated with buffer. The ether was evaporated and the residue dissolved in CHCl_3 and purified by passing through a tube containing 2 g of bone charcoal. The evaporation residue of the chloroform was a white crystalline substance: e) 1447 mg. Paper chromatography (from 200 microgrammes) revealed in b) and c) ethylpentylbarbituric acid and some barbital, but in e) no barbiturates were found, whereas in all these the bromine test was positive. Chromatography was then performed on silica gel (BDH, 8 g, column diam. 1 cm). The investigated substances were transferred into tubes in a small amount of petrol ether — ether (1 : 1) and the tubes were eluted (1) with 50 ml of petrol ether — ether (1 : 1) and then (2) with 100 ml of ether. The evaporation residues were: b) (from 68 mg) $b_1 = 38$ mg, $b_2 = 28$ mg; c) (from 60 mg) $c_1 = 28$ mg, $c_2 = 31$ mg; d) (from the total amount of 10 mg) $d_1 = 2$ mg, $d_2 = 6$ mg; e) (from 55 mg) $e_1 = 9$ mg, $e_2 = 45$ mg. In paper chromatography

ethylpentylbarbituric acid and some barbital were found in b_1 , c_1 and e_1 but the bromine test was negative. In b_2 , c_2 and e_2 the bromine test was positive but no barbiturates were found. On the other hand, in d_2 (urine) there was barbital but the bromine test was negative and in d_1 no barbiturates were found and the bromine test was negative, too. An infrared spectrum was made of all the fractions. Spectrum b_1 , Spectrum c_1 and Spectrum e_1 = *Nembutal*, Spectrum b_2 , Spectrum c_2 and Spectrum e_2 = *Carbromal*, Spectrum d_1 : *Negative*, and Spectrum d_2 = *Barbital*. — The tablets found close by were found to contain barbital (paper chromatography).

It proved, therefore, that although paper chromatography revealed in fractions b_1 , c_1 and e_1 some amount of barbital in addition to nembutal it could not be observed in the infrared spectra. An experiment was therefore made which showed that when nembutal was added to barbital the latter could be detected in the infrared spectrum when the barbital content was 5 per cent but not with certainty when the content was 2.5 per cent. Thus the barbital content of the investigated samples was very low in comparison to the nembutal and carbromal contents.

It was also observed that with the used technique of chromatography on silica gel, nembutal and with it a small amount of barbital could be isolated from carbromal in investigations b), c) and e). On the other hand, the small amount of barbital detected in investigation d) (urine) did not appear in the first fraction but only in the second fraction. In a supplementary experiment it was observed that barbital is eluted from silica gel with petrol ether — ether (1 : 1) more slowly than nembutal.

Case 29. — In the same manner as in the preceding case, and using silica gel for the final separation, it was possible to separate a) from 50 g of stomach contents, b) from 200 g of »mixed organs» and c) from 33 g of liver the fractions 1 and 2, Spectrum a_1 , Spectrum b_1 and Spectrum c_1 = *Nembutal*, and Spectrum a_2 , Spectrum b_2 and Spectrum c_2 = *Bromural*.

III. Cases with Mixture Spectra (Cases 30—31)

In these cases, also, the separation of the nonvolatile crude product was carried out with acetone.

Case 30. — The powder in the accompanying bottle gave a positive bromine test and ethylpentylbarbituric acid was detected (paper chromatography). Then a) 200 g of »mixed organs» and b) 110 ml of urine were examined and Groups I were separated and treated with buffer. After the evaporation of the ether the residue of a) weighed 128 mg and gave a positive bromine test. Of this residue 7/10 aliquots were »water purified» and 37 mg of residue was obtained after the evaporation of the ether. Additional separation yielded 15 mg of Group I_{wac}, in which ethylpentylbarbituric acid was detected (paper chromatography) and 17 mg of Group I_n, which was a viscous substance, Spectrum a = *Absorptions of both Meprobamate and Phenacetin*. Paper chromatographically (performed

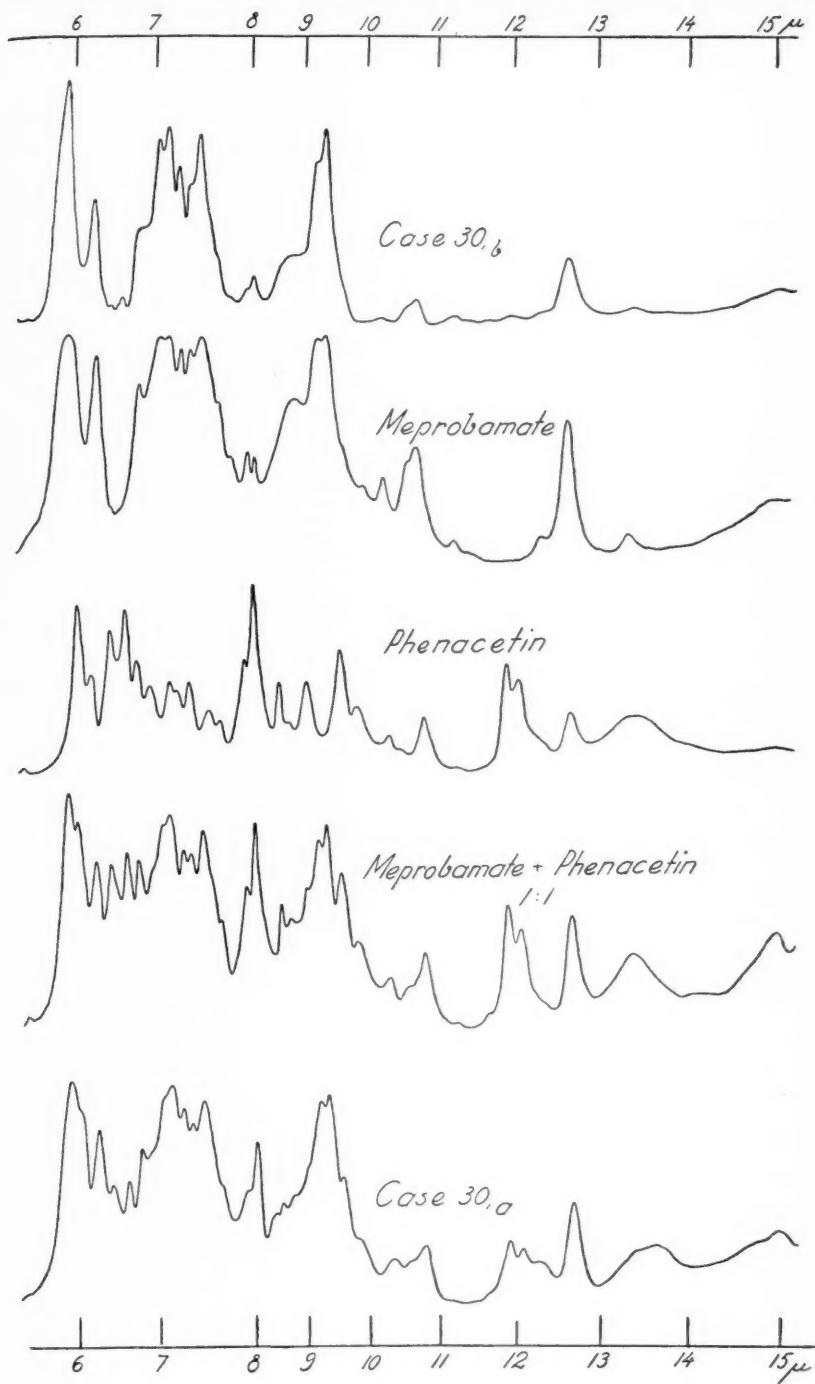


Fig. 2.

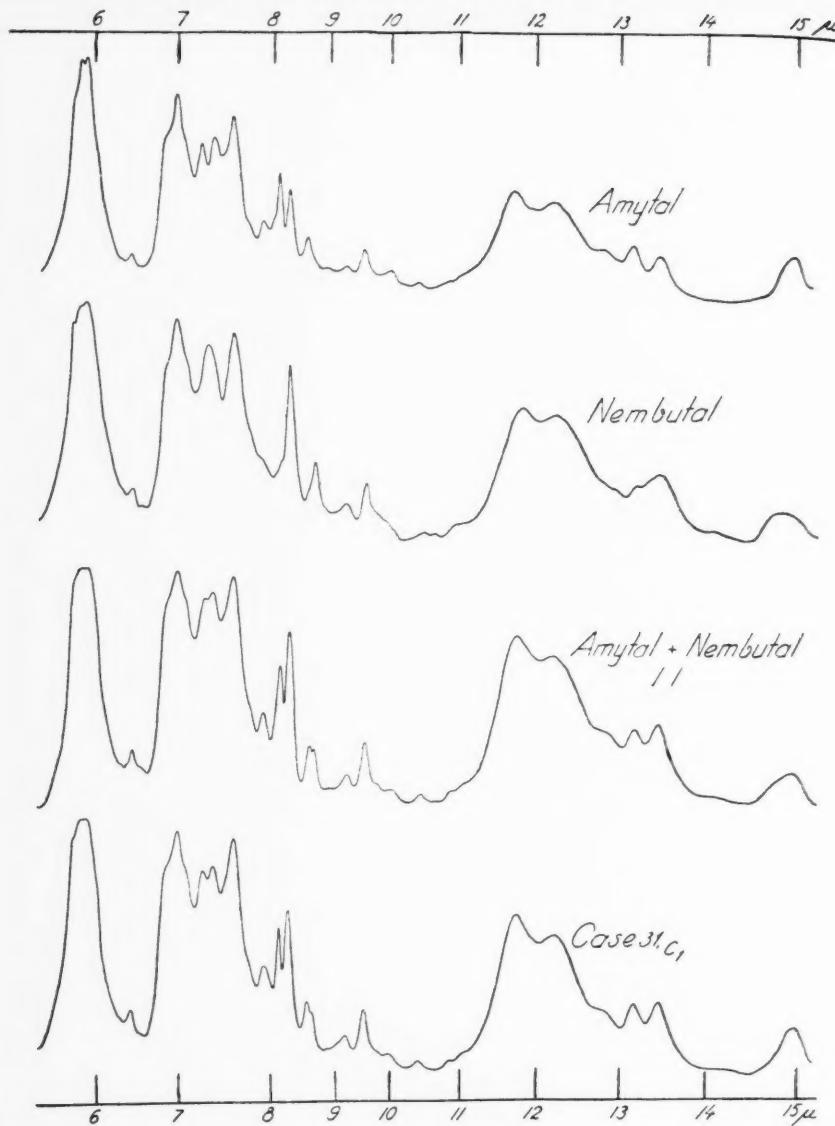


Fig. 3.

by Mrs. Elsa Hjelt, M. Sc.) the residue contained phenacetin and gave a spot of the same R_f -value as meprobamate. The residue of b) after evaporation of the ether was »water purified» and additional separation was carried out. Group I_n was 5 mg of viscous substance, Spectrum_b = *Meprobamate*. From the accompanying bottle was then separated an ether soluble neutral compound, Spectrum_c = *Phenacetin*. Fig. 2 shows the spectra of

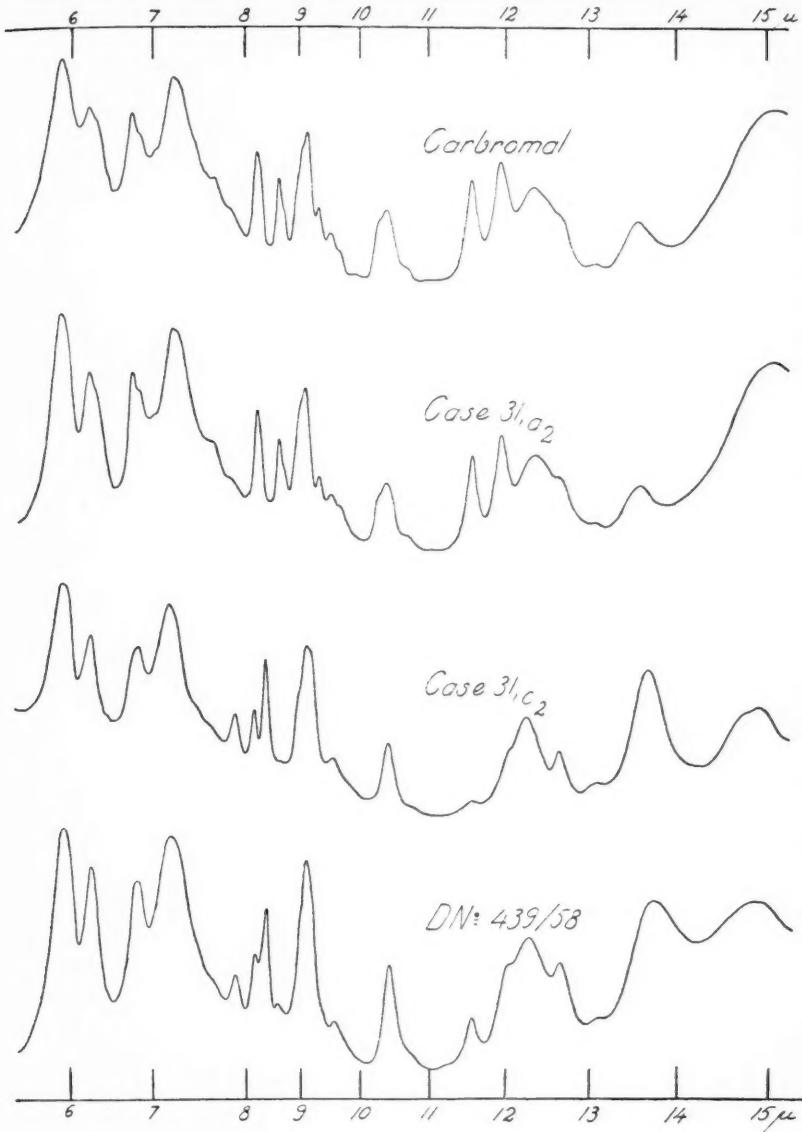


Fig. 4.

meprobamate, phenacetin and their mixture (1 : 1) as well as Spectrum_a and Spectrum_b.

Case 31. — Groups I from a) 50 g of stomach contents, b) 100 g of »mixed organs«, c) 200 g of liver, d) 400 g of brain, and e) 200 ml of urine were separated, and treated with buffer and additional separation was

carried out. The obtained 1) Groups I_{wac} and 2) Groups I_n were »water purified», yielding crystalline residues.

1) All the Groups I_{wac} gave in paper chromatography one spot of the same R_f-value as ethylpentylbarbituric acid, but Spectrum_{a₁}, Spectrum_{b₁}, Spectrum_{c₁}, Spectrum_{d₁} and Spectrum_{e₁} = *Mixture of Amytal and Nembutal*. Fig. 3 shows the spectra of amytal, nembutal and their mixture (1 : 1) and Spectrum_{e₁} (from liver).

2) All the Groups I_n gave a positive bromine test. Spectrum_{a₂} and Spectrum_{e₂} (from stomach contents and urine) = *Carbromal*, but Spectrum_{b₂} (from »mixed organs«), Spectrum_{c₂} (from liver) and Spectrum_{d₂} (from brain) all of which were found to be identical with each other, were not identical with the spectrum of carbromal although they did have many peaks similar to the latter. Fig. 4 shows the spectrum of carbromal and Spectrum_{a₂} and Spectrum_{e₂} (from stomach contents and liver).

Residue I_n separated from the brain weighed totally 6.5 mg. In the control examination the bromine test was doubtful. It could be sublimated in the range 120—170°C. The portion sublimated in the range 150—160°C had a m.p. of 191—192°C. We have earlier investigated a case of Carbral (= Nembutal + Carbromal) poisoning (D No. 439/58) in which fraction 2 separated from »mixed organs« and using silica gel for the final separation (see Cases 28 and 29) gave a spectrum identical with Spectra b₂, c₂ and d₂ now obtained by us. This Spectrum, also, is seen in Fig. 4. The substance is probably a product of carbromal metabolism¹.

COMMENTS

In endeavouring to identify a »toxic» organic substance by infrared spectroscopy from cadaverous materials, and in consideration of the minute amount of substance required for definite identification especially when an infrared microscope is used, it is obvious that the separation and purification procedures can be carried out very far at the expence of the yield. One kind of limit for these procedures however is set by the fact that a »negative» result in detection is not a negative result from the forensic-medical point of view.

When the case history indicates that one substance alone or a mixture of substances is suspected, the investigation frequently can be restricted to these substances alone, but also in these cases, and especially when a »general unknown» case is in question, when

¹ When in print we have stated that this substance is 2-ethylbutyrylurea (Preliminary Report in the Annual Meeting of the Finnish Society of Forensic Medicine April 3, 1959).

using infrared spectroscopy, one must proceed along with other methods.

The infrared spectroscopic identification of »toxic» substances calls for a large collection of spectra of substances in the field of toxicology. In most cases in the present work we have made the comparisons against spectra of known compounds taken by ourselves.

SUMMARY

1. An investigation is presented of 31 cases of suspected fatal poisoning, in which the identification of the separated substances and also the exclusion of suspected substances was carried out mainly by infrared spectroscopy.
2. The materials investigated were specimens of body organs taken at autopsy, and medicines, etc. left by the deceased.
3. The methods of separation and purification are described. In the separation of volatile substances, one of the techniques used was steam distillation after precipitation with acetone. Nonvolatile substances were separated with acetone and the crude product obtained was fractionated into four groups. In the group of acid and neutral substances »water purification» was used, and when necessary the separation of neutral substances and fractionation of acid substances into two groups using buffer pH 7.2 was done. The further treatment of the »fatty mass» obtained by acetone treatment is described in two cases, in which Carbromal and Mesantoin were separated. Further procedures of purification and separation were sublimation (sublimation on a KBr disc.), ion exchange and chromatography on silica gel, which latter allowed the separation of Nembutal and Carbromal, and of Nembutal and Bromural.
4. Techniques of taking infrared spectra are presented. From crystals the spectrum is readily obtained using infrared microscope when crystals are placed on a thin KBr disc.
5. Using the infrared spectra the following substances separated from cadaverous materials were identified:
a) Trichloroethylene (2 cases), Parathion (3 cases), p-nitrophenol, DDT (?), Systox;
b) Phenacetin (3 cases), Megimide, Meprobamate (2 cases), Valamin, Mesantoin, Bromural (3 cases), Carbromal (2 cases), Sedormid;
c) Butobarbitone, Phenobarbital, Nembutal (2 cases), Barbital;
d) Aminopyrine, Chloroquine (2 cases), Quinine, Quinidine, Morphine.

6. Mixtures of Meprobamate-Phenacetin and Amytal-Nembutal were detected in two spectra.

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FROM THE MEDICAL DEPARTMENT OF THE MARIA HOSPITAL, HELSINKI,
AND THE DEPARTMENT OF ANATOMY, UNIVERSITY OF HELSINKI

MAST CELLS IN THE GASTRIC MUCOSA OF PATIENTS WITH PEPTIC ULCER AND GASTRIC CANCER

by

M. SUNDBERG and M. SIURALA

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The present writers (3) have previously found a satisfactory correlation between the mast cell count and the morphological condition of the gastric mucosa in biopsy specimens taken from the body mucosa of patients not suffering from peptic ulcer or gastric cancer. The mast cell count was found to be significantly greater in specimens from cases with gastritis than in those with a normal gastric mucosa. However, in some cases the mast cell count was the same in specimens taken from the same patients although the morphological condition of the mucosa was quite different. This suggests that also other factors than the morphological state of the gastric mucosa might influence the mast cell count of the gastric mucosa.

The aim of this study was to investigate whether the presence of peptic ulcer or gastric cancer has any influence upon the mast cell count of the gastric mucosa.

MATERIAL AND METHODS

The series consisted of 30 patients. In 12 cases the specimens were obtained from resected parts of the stomach and in the remaining 18 cases by means of the suction biopsy method (35 specimens). The specimens were always taken outside the ulcer and cancer area.

The specimens were fixed in 4% fresh lead acetate solution, cut into 10 μ slices and treated with alcohol and stained with 1% toluidine blue aqueous solution. A Zeiss binocular microscope with objective No. 40, ocular No. 10, was used. The visual field was 0.04 sq.mm. The cells were counted in alternate slices and only cells with visible nuclei were noted. Mast cells were counted from a region above and adjacent to the muscularis mucosae and the visual field was moved along the upper border of the muscularis mucosae. Only specimens with a well preserved muscularis mucosae were examined. The number of mast cells was determined in 2 sq.mm. The cell count per sq.mm. was calculated from the result obtained. One of us counted the mast cells and the other made the gastric biopsies and histological examinations.

21 of the cases were men and 9 women. 14 had duodenal ulcer, 8 gastric ulcer and 8 gastric cancer. The mean age of the patients was 54 years. Normal gastric mucosa was encountered in 14 cases and atrophic gastritis (significant loss of normal body glands) in the remaining 16 cases. Superficial and interstitial gastritis were found only in connection with atrophic gastritis. The T-test was used for the statistical calculations.

RESULTS

The mean mast cell count in duodenal ulcer, gastric ulcer and gastric cancer in relation to the morphological condition of the gastric mucosa is presented in Table 1, and the distribution of the individual cases in Figure 1.

According to Table 1, the mean mast cell count in gastric cancer (140.5 cells per sq.mm.) was markedly higher than in duodenal (89.8) and gastric ulcer (90.8). The difference was statistically significant ($p < 0.01$). On the other hand, all the cases with gastric cancer had atrophic gastritis of considerable degree and in peptic ulcer cases with a normal gastric mucosa the mean mast cell count was markedly lower than in peptic ulcer cases with atrophic gastritis. The mean mast cell count in all the cases with normal gastric mucosa was 70.5 cells per sq.mm. and that of cases with atrophic gastritis 132.3. The difference was statistically highly significant ($p < 0.001$). As mentioned before, superficial gastritis was found only in connection with atrophic gastritis.

TABLE 1

THE MEAN MAST CELL COUNT OF THE GASTRIC MUCOSA IN DUODENAL ULCER,
GASTRIC ULCER AND GASTRIC CANCER ACCORDING TO THE CONDITION OF THE
GASTRIC MUCOSA

State of the Gastric Mucosa	Mean Mast Cell Count per sq.mm.			
	Duodenal Ulcer	Gastric Ulcer	Gastric Cancer	Total
Normal	68.0	76.7	—	70.5 ± 8.6
Gastritis (atrophic)	144.2	103.2	140.5	132.3 ± 10.8
Total	89.8 ± 16.3	90.8	140.5 ± 6.4	

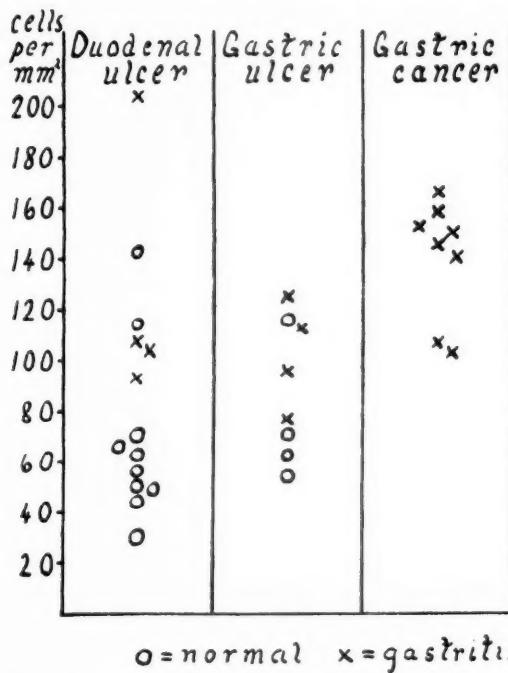


Fig. 1. -- Mast cell counts in the gastric mucosa in duodenal ulcer, gastric ulcer and gastric cancer according to the condition of the gastric mucosa.

The mean mast cell count in specimens taken by suction biopsy was 98.6 and in those obtained by resection 100.8 cells per sq.mm., *i.e.* of the same order of magnitude. The different groups revealed also only slight and insignificant differences in this respect. Thus, in duodenal ulcer specimens with a normal gastric mucosa the mean mast cell count in suction biopsy specimens was 72.2, in resection specimens 62.0 cells per sq.mm.

DISCUSSION

The mast cell count was found to be significantly higher in gastric cancer than in peptic ulcer. On the other hand, there was a highly significant difference in the mast cell count between cases with normal gastric mucosa and those with gastritis. This is in agreement with the findings of Siurala and Sundberg (3) for the mast cell count in the gastric mucosa of patients not suffering from peptic ulcer or gastric cancer. The mean mast cell count in gastric cancer (140.5 ± 6.4), in which atrophic gastritis was found in all the specimens, was of the same order of magnitude as that found by Siurala and Sundberg in atrophic gastritis cases without gastric cancer (150 ± 13.7). These results suggest that the mast cell count in gastric cancer is dependent upon the presence of gastritis rather than upon the cancer itself. In peptic ulcer cases with normal gastric mucosa the mean mast cell count was 70.5 cells per sq.mm., *i.e.* of the same order as in the cases with normal gastric mucosa (79 cells per sq.mm.) reported by Siurala and Sundberg. The mean mast cell count in duodenal ulcer cases with gastritis was 144.2, *i.e.* markedly higher than in these with normal mucosa.

The results obtained above contradict those obtained by Janes and McDonald (1) and Räsänen (2). Janes and McDonald found that the mast cell count was higher in peptic ulcer than in cancer cases. Räsänen, counting the mast cells from the whole gastric mucosa, found in resection specimens (peptic ulcer and gastric cancer cases) that the mast cell count was higher in connection with a normal gastric mucosa than with gastritis.

We later checked our results by counting the mast cells from the whole gastric mucosa of the same specimens, moving the objective from the muscularis mucosae towards the surface

epithelium. With this method the mean mast cell count of normal specimens was 79.8 and that of specimens with atrophic gastritis 105.3 cells per sq.mm. Although these values differed to some extent from those previously obtained, the mean mast cell count of specimens with atrophic gastritis was nevertheless clearly higher than that of normal specimens.

SUMMARY

The mast cell count of the gastric mucosa was studied in suction biopsy specimens (18 cases) and in specimens obtained from resected parts of stomachs (12 cases) of 14 duodenal ulcer, 8 gastric ulcer and 8 gastric cancer cases. Normal gastric mucosa was encountered in 14 and atrophic gastritis in 16 cases. The mean mast cell count in duodenal ulcer was 89.9, in gastric ulcer 90.8 and in gastric cancer 140.5 cells per sq.mm. The difference in the mast cell count between gastric cancer and peptic ulcer was statistically significant. On the other hand, the mast cell count in connection with atrophic gastritis was significantly higher ($p < 0.001$) than with normal gastric mucosa, and atrophic gastritis occurred in every case of gastric cancer. The results suggest that the difference in the mast cell count between gastric cancer and peptic ulcer cases is due to the different mucosal condition in these groups. There was no significant difference in the mast cell count between the resected specimens and those obtained by means of the suction biopsy tube.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY,
UNIVERSITY OF HELSINKI

INHERITANCE OF THE Gm SERUM GROUP

by

O. MÄKELÄ¹ and ANJA THILIKAINEN

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In 1956 Grubb and Laurell (4) introduced a new Mendelian character of man, the Gm (gamma-globulin) group. They showed that the agglutination by certain rheumatoid arthritic sera of group O Rh positive red cells coated with selected incomplete anti-Rh antibodies could be inhibited by certain but not all human sera. The inhibitor was located in the gamma-globulin fraction.

On the basis of the family investigations the authors came to an assumption of autosomal, unifactorial inheritance. The phenotypes were called Gm(a+) (inhibitory) and Gm(a-) (not inhibitory). The corresponding genes were called Gm^a (dominant) and Gm (recessive). The last symbol without a subscript denotes the gene(s) at the same locus not being Gm^a. The Gm group has not been found to be correlated with sex, the ABO, MNS, Rh, P, Lewis, Kell, Lutheran or Duffy blood groups, nor with the Hp group (4, 5).

Grubb (2, 3) has published two reviews of the present status of study in this field. These papers contain complete reference lists. The observations of the inheritance of Gm group reported in this paper are in accordance with the original assumptions of Grubb and Laurell.

METHODS

A slightly modified form of the technique described by Grubb and Laurell (4) has been used throughout.

¹) Aided by a grant from State Comission on Natural Science.

Diluent. — For dilutions and washing of the red cells a saline solution of pH 7.2 (3 parts NaCl physiol. + 1 part M/15 Sörensen's phosphate buffer) was used.

Red Cells. — The red cells used were of group O CcDEe and they were preserved at +4°C for not more than 3 days.

Serum Containing Incomplete Anti-D. — For the sensitization of the red cells a single serum containing anti-Rh_o (D) was used throughout. The above test cells, when treated with trypsin, were agglutinated by it up to the dilution of 1: 256 while untreated ones in saline medium were not agglutinated. The serum was preserved at -20°C.

Rheumatoid Arthritic Sera. — Two selected rheumatoid arthritic sera known to agglutinate Rh sensitized red cells and not to agglutinate uncoated Rh positive erythrocytes were used for every serum sample studied for inhibition. As the one serum in the course of the whole work use was made of serum 45, which agglutinated sensitized cells up to the titre 1: 320. As the other we used either of the samples given to us by Dr Grubb; their titres ranged from 320 to 640.

Coating of Red Cells with Anti-D. — To 0.2 ml of thrice washed packed cells was added 2.5 ml of anti-D serum diluted 1: 10. It was then incubated at 37°C for 2 hours, and the cells were washed four times. The cells thus sensitized were agglutinated strongly by selected rheumatoid arthritic sera, and the agglutination was inhibited by pooled human gamma-globulin and by the sera of persons known to be Gm(a+).

Agglutination Test. — From the rheumatoid arthritic sera dilutions from 1: 5 to 1: 1280 were made. 0.1 ml of the serum dilutions and 0.1 ml of a 0.4% suspension of the sensitized red cells were put to test tubes. The results were read after 4—6 hours.

Agglutination-inhibition Test (Gm Grouping)

The general technique is the same as in agglutination test. The serum whose Gm group it was desired to determine was diluted with a buffer solution so as to obtain the concentrations 1: 4, 1: 12, and 1: 36. Of each of these dilutions 0.05 ml was measured into a test tube, into which another 0.05 ml of known rheumatoid arthritic serum was mixed at a dilution of 1: 15 (10 to 20 agglutinating doses). After a quarter of an hour, 0.1 ml of a 0.4% suspension of the sensitized red cells was added into each tube. As controls were used: 1) 0.05 ml of each serum to be examined at a dilution of 1: 4 together with 0.05 ml of the buffer solution, 2) 0.05 ml of the rheumatoid arthritic sera used, at the working dilutions plus 0.05 ml of the buffer solution, 3) 0.1 ml of the buffer solution. Into each control tube was added 0.1 ml of sensitized cell suspension.

Along with the samples to be studied a simultaneous test series was made daily of three persons on the laboratory staff, one of whom was Gm(a+), another giving a weak Gm(a+) reaction, and the third Gm(a-).

MATERIAL

The series consisted of three groups of persons: 1) unrelated Finnish blood donors, 2) families consisting of the parents and at least one child, and 3) monozygous and dizygous twins.

In the calculations of gene frequencies parents and one twin of each pair (picked out at random) has been included. Considerable part of the families was from the Åland islands the origin of whose Swedish-speaking population is uncertain. Parents of these families have not been included in these calculations for the Finnish population. The Finnish gene frequencies obtained were, however, used in the treatment of the family material because: 1) Finnish, Swedish (4) and Danish (5) do not seem to differ appreciably from one another, and 2) the gene frequencies of the series consisting of the parents of the Åland families did not differ markedly from the Fennoscandinavian frequencies, either.

The family series comprised 84 families with 263 children tested (all being more than 1 years of age).

The twin series was a part of the material collected by Oy Alkoholiliike Ab and comprising Finnish male twins born between years 1920 and 1929. Out of this series all such pairs were included as showed a high concordance in the polysymptomatic similarity tests and identical blood groups (ABO, MN, Rh) and haptoglobin groups. About an equal number of unidentical pairs (some differences in blood groups) were picked for testing.

RESULTS

Of the 477 unrelated Finnish persons 310 were Gm(a+), 167 Gm(a-) and 8 intermediate types. The last group is not included in the calculations of gene frequencies. The phenotype frequencies thus are: Gm(a+) 0.6499 and Gm(a-) 0.3501. Assuming the phenotype Gm(a-) to represent the genotype GmGm the gene frequencies are: for the gene Gm $\sqrt{0.3501} = 0.592$ and for the gene Gm^a $1 - \sqrt{0.6499} = 0.408$.

The Gm group did not seem to be correlated with sex or with ABO or Rh_o (D) blood group.

Tables 1—9 give the results of the treatment of the family material. The agreement between observed and expected incidence

TABLE 1
OBSERVED AND EXPECTED DISTRIBUTION OF MATING TYPES

Mating Type	No. Obs.	No. Exp.	χ^2
Gm(a+)xGm(a+)	42	35.5	1.20
Gm(a+)xGm(a-)	33	38.2	0.71
Gm(a-)xGm(a-)	9	10.3	0.16
Total no. of matings	84	Total χ^2	2.07 (d.f. = 2) 0.50 > P > 0.30

TABLE 2
OBSERVED DISTRIBUTION OF FAMILIES OF MATING TYPE Gm(a+)xGm(a-)

No. of Children in Family of Group Gm(a-)	No. of Children in Family Tested for Gm Group					Total
	1	2	3	4	5	
0	6	4	3	4	0	17
1	2	4	2	0	1	9
2	0	2	1	1	1	5
3	0	0	0	0	1	1
4				1		1
Total no. of families	8	10	6	6	3	33
Total no. of families with at least one Gm(a-) child	2	6	3	2	3	16

(Figures in cells indicate no. of families)

TABLE 3
MATING TYPE Gm(a+)xGm(a-)
ANALYSIS OF FAMILIES WITH AT LEAST ONE Gm(a-) CHILD

No. of Children in Family	No. of Families	No. of Gm(a-) Children		
		Obs.	Exp.	Variance
1	2	2	2.000	0.000
2	6	8	7.998	1.332
3	3	4	5.142	1.470
4	2	6	4.266	1.564
5	3	6	7.743	3.246
Total	16	26	27.149	7.612

$\chi^2 = 0.173$ (d.f. = 1) $0.70 > P > 0.50$

TABLE 4
MATING TYPE Gm(a+)xGm(a—)
ANALYSIS OF FAMILIES WITH AND WITHOUT CHILDREN OF GROUP Gm(a—)

No. of Children in Family	No. of Families	No. of Families with at Least One Gm(a—) Child		
		Obs.	Exp.	Variance
1	8	2	2.974	1.868
2	10	6	5.576	2.467
3	6	3	3.903	1.364
4	6	2	4.184	1.266
5	3	3	2.161	0.604
Total	33	16	18.798	7.569

$$\chi^2 = 1.034 \quad (\text{d.f.} = 1) \quad 0.50 > P > 0.30$$

TABLE 5
OBSERVED DISTRIBUTION OF FAMILIES OF MATING TYPE Gm(a+)xGm(a+)

No. of Children in Family of Group Gm(a—)	No. of Children in Family Tested for Gm Group								Total
	1	2	3	4	5	6	7	8	
0	1	5	11	2	4	2	1	1	27
1	3	4	2		1				10
2		1	2	2					5
Total no. of families	1	8	16	6	6	3	1	1	42
Total no. of families with at least one Gm(a—) child	3	5	4	2	1				15

(Figures in cells indicate no. of families)

TABLE 6
MATING TYPE Gm(a+)xGm(a+)
ANALYSIS OF FAMILIES WITH AT LEAST ONE Gm(a—) CHILD

No. of Children in Family	No. of Families	No. of Gm(a—) Children		
		Obs.	Exp.	Variance
1	0	0	0.000	0.000
2	3	3	3.429	0.366
3	5	6	6.485	1.315
4	4	6	5.852	1.680
5	2	4	3.278	1.184
6	1	1	1.825	0.776
Total	15	20	20.869	5.321

$$\chi^2 = 1.419 \quad (\text{d.f.} = 1) \quad 0.25 > P > 0.20$$

TABLE 7
MATING TYPE $Gm(a+)$ $\times Gm(a+)$
ANALYSIS OF FAMILIES WITH AND WITHOUT CHILDREN OF GROUP $Gm(a-)$

No. of Children in Family	Total No. of Families	No. of Families with at Least One $Gm(a-)$ Child		
		Obs.	Exp.	Variance
1	1	0	0.138	0.119
2	8	3	1.937	1.468
3	16	5	5.112	3.479
4	6	4	2.269	1.411
5	6	2	2.530	1.463
6	3	1	1.363	0.744
7	1	0	0.479	0.249
8	1	0	0.497	0.250
Total	42	15	14.325	9.183

$\chi^2 = 0.050$ (d.f. = 1) $0.99 > P > 0.98$

TABLE 8
DISTRIBUTION OF FAMILIES OF MATING TYPE $Gm(a-)$ $\times Gm(a-)$

No. of Children in Family Tested	Observed No. of Families	Total No. of Children of Group	
		$Gm(a+)$	$Gm(a-)$
1	1	0	1
2	1	0	2
3	5	0	15
4	2	0	8
Total	9	0	26

TABLE 9
SUMMARY OF TESTS ON THE FAMILY SERIES

Test	Mating	χ^2	d.f.
No. of $Gm(a-)$ children, given m_c	$Gm(a+) \times Gm(a-)$	0.173	1
No. of $Gm(a-)$ children, given M_c	$Gm(a+) \times Gm(a+)$	1.419	1
Sum of m_c , given n_c	$Gm(a+) \times Gm(a-)$	1.034	1
Sum of M_c , given N_c	$Gm(a+) \times Gm(a+)$	0.050	1
Total		2.676	4

$0.70 > P > 0.50$

Symbols: m_c = total number of families of mating type $Gm(a+) \times Gm(a-)$.

M_c = No. of families of mating type $Gm(a+) \times Gm(a-)$ with at least one $Gm(a-)$ child.

n_c = total number of families of mating type $Gm(a+) \times Gm(a+)$.

N_c = No. of families of mating type $Gm(a+) \times Gm(a+)$ with at least one $Gm(a-)$ child.

of various mating types is satisfactory. Two children of $Gm(a+)$ \times $Gm(a-)$ families were $Gm(a\pm)$. They are not included in the tables. The Tables 2—9 are drawn according to the method described by Smith (6), which takes into consideration the fact that say the matings $Gm(a+) \times Gm(a-)$ can be either $Gm^aGm^a \times GmGm$ or $Gm^aGm \times GmGm$.

The matings in which there is at least one $Gm(a-)$ child (disclosing the genotype of the parents) are analysed separately in Tables 3 and 6. In Tables 4 and 7 it is tested whether there is a satisfactory division between matings with and without recessive children, taking the gene frequencies in account. In Table 8 the families of the mating type $Gm(a-) \times Gm(a-)$ are tabulated. All the children are $Gm(a-)$ as expected. The results of all tests are summarized in Table 9. The agreement is satisfactory.

TABLE 10
THE GM GROUPS IN A TWIN SERIES (85 PAIRS)

	Gm Groups Identical	Gm Groups Different	Total
Presumably monozygous twins (')	obs. 37 pairs exp. 37 pairs	obs. 0 pairs exp. 0 pairs	37 pairs
Dizygous twins	obs. 36 pairs exp. 35.7 pairs (")	obs. 12 pairs exp. 12.3 pairs (")	48 pairs

* i.e. of identical sex, blood groups, Hp groups and of high concordance in the similarity test.

" The calculation of the expected numbers is based on the gene frequencies.

The findings in the twin series (Table 10) support the hypothesis of a hereditary nature of Gm group. It may be mentioned that twins of one of the identical twin pairs belonged to the «intermediate» type.

MISCELLANEOUS OBSERVATIONS

In the family series there were 17 children between 1 and 4 years. The phenotypes of this series did not appreciably differ from those of the material comprising the eldest children of the corresponding families (Table 11). The phenotypes of these infants also differed from the mother's phenotype (3 cases) as often as from the father's (3 cases).

TABLE 11

THE GM GROUP OF THE CHILDREN BETWEEN 1 AND 4 YEARS AND OF THEIR RESPECTIVE ELDEST SISTERS

	The Number of Small Children	The Number of the Eldest Sisters (age ≥ 5 years)
Gm(a+)	14	12
Gm(a--)	3	5

DISCUSSION

Linnet-Jepsen *et al.* (5) have discussed the value of the Gm groups in cases of disputed paternity.

The use of the Gm groups in disputed paternity cases is hindered by the fact that the newborn infant always appears to possess his mother's phenotype. It is not known at what age the group corresponding to the child's genotype generally manifests itself. Judging by this work it seems to have taken place in most cases by the end of the 1st year. False exclusions cannot be caused by the child having his mother's »Gm substance» nor by the child's own substance having not had time to develop because of the early age, since the only combination of exclusion is: mother Gm (a-), child Gm(a+). On the contrary, it is possible that possibilities of exclusion would be lost for the latter reason but even of this there does not seem to be much risk after the child has passed his first year.

SUMMARY

The frequency of the gene Gm^a in the Finnish population was found to be 0.408. The distribution of Gm groups in 84 families (263 children) studied is in accordance with the assumption of Grubb and Laurell (4) of autosomal unifactorial inheritance of the Gm group. All 37 pairs of monozygous twins studied had identical Gm groups.

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PROTEOLYTIC ENZYMES OF THE PLEURAL AND PERITONEAL FLUIDS IN VARIOUS PATHOLOGICAL CONDITIONS

by

TEPPO VARTIO

(Received for publication January 30, 1959)

The knowledge of the enzymes of the pleural and peritoneal fluids may lead to a clearer understanding of the physiology of the organs in question and may also be of clinical value in diseases of these organs. Therefore a study was made of the three proteolytic enzymes, pepsin, cathepsin, and trypsin, of pleural and peritoneal fluids in various pathological conditions.

MATERIAL AND METHODS

Proves were taken from the pleural effusion and from the ascitic fluid of patients suffering from various diseases (table 1). From the same sample the three proteolytic enzymes were determinated. The determinations were made by the following methods:

Cathepsin: The cathepsin was determinated according to the method for urocathepsin determination of Buchs (1) modified for requirement of determining from the in many cases originally turbid specimen. Thus the method formed as follows:

0.5 Gm of edestin (Hoffmann-La Roche, Basel) was dissolved in 100 cc. of glycocoll-HCl buffer pH 3.3 (85 cc. 0.1 N glycocoll + 15 cc. 0.1 N HCl). 5 cc. of this buffer + 1 cc. of the specimen to be examined were incubated in a 37°C waterbath for one hour. For the determination of the breaking up of the edestin 1 cc. of the above mixture was mixed with 8 cc. of 0.5

per cent gummi-arabicum solution and 1 cc. of 20 per cent sulfosalicylic acid and the turbidity caused by the sulfosalicylic acid and stabilized by the gummi-arabicum solution was read in E.E.L.-photometer with blue filter. The control-tube for the sake of comparison was made in the same manner, but the precipitation and the reading in photometer was performed immediately. The fact, that the reading of this tube didn't change noteworthy during one hour was verified repeated times. The amount of edestin broken up by the fluid to be examined was calculated from the difference between the readings in photometer of these two tubes. The cathepsin activity of the specimen was expressed in mg:s of edestin broken up by one ml of the specimen in an hour.

Pepsin. — The pepsin determination was made exactly in the same manner except that the buffer solution was made acid to the methyl orange, *i.e.* pH 3 or less, by adding sufficient amount of 2 N HCl. The pepsin activity was expressed in mg:s of edestin broken up by one ml of the specimen in an hour.

Trypsin. — As the edestin was not dissolved in alkaline medium, the protein used in this experiment was dried human serum. The buffer solution, pH 9.5, was prepared from NH₄Cl and NH₃. To obtain a sufficient precipitation, 4 cc. instead of 1 cc. of sulfosalicylic acid was used, and this was taken into account in the final calculation. For the rest the determination was made in the same manner as the foregoing determinations. The trypsin activity was expressed in mg:s of protein broken up by one ml of the specimen in an hour.

By the reading in photometer the additional turbidity caused by the in many cases originally turbid specimen was taken into account. Thus in these cases the standard-curve was drawn separately for each determination with the help of the control-tube (containing 0.04 per cent of edestin in pepsin and cathepsin determination, and 0.03 per cent of protein in trypsin determination) and the 0-tube with the specimens own turbidity. That the reading in this 0-tube didn't change noteworthy if the precipitation was made immediately or after being in a 37°C waterbath for one hour, was verified repeated times. Thus the amount which the fluid possible breaks up from its own proteins, is minimal.

RESULTS

The results appear from table 1. From the three proteolytic enzymes the pepsin seemed to exist in greater amounts than the other two of the specimens. The highest values of pepsin, 0.72 and 0.75 mg edestin/ml, were found in one case of pulmonary cancer and in one case of hydrothorax. The cathepsin was in most cases 0, and the highest value, 0.57 mg edestin/ml, was in one case of exsudative pleurisy. The trypsin was in most cases 0, and the highest value, 0.35 mg protein/ml, was verified in one case of pleural empyema.

TABLE 1.
PEPSIN, CATHEPSIN AND TRYPSIN IN PLEURAL AND PERITONEAL FLUIDS IN
VARIOUS DISEASES

Age	Sex	Pepsin mg Edestin/ml	Cathepsin mg Edestin/ml	Trypsin mg Protein/ml	Notes
<i>Pleural Fluid</i>					
<i>Pleuritis exsudativa:</i>					
30	m	0.32	0.00	0.12	
54	,	0.30	0.27	0.00	
29	,	0.15	0.00	0.07	
41	,	0.00	0.00	0.00	
40	,	0.00	0.00	0.00	
58	,	0.07	0.10	0.00	
25	,	0.02	0.57	0.15	
26	,	0.35	0.00	0.00	
44	f	0.00	0.00	0.00	
47	,	0.20	0.00	0.00	
60	,	0.20	0.00	0.10	
<i>Pleuropneumonia:</i>					
46	m	0.50	0.00	0.00	
34	,	0.00	0.25	0.05	
25	f	0.50	0.00	0.00	
<i>Empyema pleurae:</i>					
47	m	0.00	0.07	0.00	
66	,	0.22	0.30	0.35	
<i>Carcinoma pulmonum:</i>					
56	m	0.72	0.35	0.00	Bloody specimen
60	,	0.00	0.12	0.00	
58	,	0.00	0.00	0.05	
61	,	0.50	0.27	0.05	
<i>Hydrothorax:</i>					
66	m	0.75	0.00	0.17	
62	f	0.20	0.00	0.00	
39	,	0.30	0.00	0.00	
<i>Peritoneal Fluid</i>					
<i>Cirrhosis hepatis:</i>					
60	m	0.30	0.35	0.00	
52	f	0.25	0.00	0.00	
43	,	0.15	0.05	0.00	
<i>Ca. cavi abd. c. metast.:</i>					
52	f	0.50	0.00	0.00	
60	,	0.25	0.00	0.00	
47	,	0.50	0.00	0.00	
61	,	0.35	0.02	0.05	
<i>Hepatoma:</i>					
24	m	I 0.00	0.00	0.00	Bloody specimen
		II 0.00	0.17	0.00	
<i>Peritonitis tuberculosa:</i>					
40	m	0.00	0.00	0.00	
<i>Insufficiencia cordis:</i>					
39	f	0.30	0.05	0.00	

COMMENTS

The amount of the three proteolytic enzymes in pleural and peritoneal fluids in various diseases didn't seem to have any relation to the amount of leucocytes in the fluid. *E.g.* in one case of pleural empyema with heavy pus only cathepsin showed traces of activity. Thus we may assume that these enzymes do not originate from leucocytes but through diffusion from blood, otherwise than the case has been supposed to be *e.g.* in the cerebrospinal fluid (2, 3). This could also declare the great variability of the presence of these enzymes in the pleural and peritoneal fluids, as the blood pepsin and cathepsin are known to show very great variability in their quantity (4) depending on the occasional state of their gastric source. Another explanation of this variability could be the variable content of albumin in the fluids, as the proteolytic enzymes are known to be like albumins, and thirdly the freshness of the effusion. The presence of antitrypsin in the blood (3) could be an explanation to the very small amounts of trypsin in the specimens studied.

As there seem to be no characteristic changes in the proteolytic enzyme activities of the pleural and peritoneal fluids in various diseases, it seems that the determination of the three enzymes in the diseases studied has no value in diagnostical aspect.

SUMMARY

The three proteolytic enzymes, pepsin, cathepsin, and trypsin, were studied from the pleural and peritoneal fluids in various diseases. Little amounts of these enzymes were found in some specimens and from these the pepsin seemed to exist in greater amounts than the other two, and trypsin was present only in few cases and in very little amounts. No characteristic changes in various diseases could be verified.

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FROM THE MEDICAL DEPARTMENT OF THE PROVINCIAL HOSPITAL, OULU

PROTEOLYTIC ENZYMES IN CEREBROSPINAL FLUID IN NORMAL AND CERTAIN PATHOLOGICAL CONDITIONS

by

TEppo VARTIO

(Received for publication January 30, 1959)

To obtain a clearer understanding of the physiology of the central nervous system and to find methods, which could be of any clinical value in diseases of the central nervous system, the proteolytic enzymes of cerebrospinal fluid have been studied by a number of workers. The enzymes studied have been: trypsin (1, 2, 3), cathepsin (1), aminopolypeptidase (1), dipeptidase (1), and peptidases (5). In normal cerebrospinal fluids no proteolytic activities have been found, except in some proves tripeptidases (5). In various pathological conditions some activities of these enzymes have been found, especially in tuberculous meningitis (2, 5). The source of enzymes in these cases has been discussed. This has been assumed to be due to the increased number of leucocytes (1, 2, 5) or to increased meningeal permeability (2, 3). The purpose of the following study is to examine the three main proteolytic enzymes, pepsin, cathepsin, and trypsin, in cerebrospinal fluid in normal and certain pathological conditions, and compare the presence of these enzymes with each other.

MATERIAL AND METHODS

Proves were taken from the cerebrospinal fluid of 21 persons, in which in subsequently examinations no pathological changes could be found, and from 22 patients suffering from various diseases

in the central nervous system (table 1). From the same sample the three proteolytic enzymes were determined. The determinations were made by the method described earlier (6). The amount of fluid used in the tests was usually 0.5 cc. instead of 1.0 cc., and this was taken into account in the final calculation.

RESULTS

The results appear from table 1. In normal cerebrospinal fluids the pepsin and cathepsin showed some activity in some cases, and the trypsin was 0 in all cases. In various pathological conditions the pepsin and cathepsin showed some activity in most cases, and the trypsin showed traces of activity in a few cases.

COMMENTS

The amount of the three proteolytic enzymes in cerebrospinal fluids in various diseases didn't seem to have any relation to the amount of leucocytes in the fluid (See the enzymes *e.g.* in the cases of purulent meningitis). This might support the opinion, that these enzymes do not originate from leucocytes, but through diffusion from blood. This could also declare the great variability of the presence of these enzymes in the cerebrospinal fluids, as the blood pepsin and cathepsin are known to show very great variability in their quantity (4) depending on the occasional state of their gastric source. Another explanation of this variability could be the variable content of albumin in the fluid, as the proteolytic enzymes are known to be like albumins, and thirdly the freshness of the pathologic changes in the meninges. The presence of antitrypsin in the blood and cerebrospinal fluid (2) could be an explanation to the very little amounts of trypsin in the specimens studied.

As there seem to be no characteristic changes in the proteolytic enzyme activities of the cerebrospinal fluid in various diseases, it seems that the determination of the three enzymes in the diseases studied has no value in diagnostical aspect.

TABLE 1
PEPSIN, CATHEPSIN AND TRYPSIN IN CEREBROSPINAL FLUID IN NORMAL AND CERTAIN PATHOLOGICAL CONDITIONS

Age	Sex	Pepsin mg Edestin/ml	Cathepsin mg Edestin/ml	Trypsin mg Protein/ml	Notes
<i>Normals</i>					
68	m	0.00	0.20	0.00	
43	♂	0.20	0.00	0.00	
40	♂	0.00	0.00	0.00	
60	♀	0.00	0.03	0.00	
58	♀	0.00	0.15	0.00	
24	♂	0.00	0.00	0.00	
33	♀	0.00	0.00	0.00	
42	♀	0.20	0.17	0.00	
52	f	0.00	0.02	0.00	
70	♂	0.25	0.00	0.00	
58	♀	0.12	0.00	0.00	
40	♀	0.00	0.00	0.00	
48	♂	0.10	0.10	0.00	
48	♀	0.00	0.20	0.00	
34	♂	0.25	0.00	0.00	
56	♂	0.18	0.05	0.00	
61	♂	0.00	0.05	0.00	
22	♂	0.00	0.00	0.00	
20	♂	0.00	0.15	0.00	
18	♂	0.00	0.07	0.00	
17	♂	0.00	0.12	0.00	
<i>Haemorrhagia subarachnoidalis</i>					
63	m	0.20	0.00	0.00	
78	♂	0.00	0.22	0.00	
45	♀	0.15	0.07	0.00	
49	♂	0.10	0.00	0.00	
16	f	0.10	0.00	0.00	
64	♂	0.10	0.00	0.00	
34	♀	0.15	0.07	0.10	
45	♀	0.10	0.00	0.00	
28	♂	0.05	0.35	0.10	
70	♂	0.00	0.07	0.00	
28	♂	0.00	0.85	0.10	
<i>Meningitis tuberculosa</i>					
56	m	0.15	0.15	0.00	
40	♂	0.07	0.00	0.00	
27	f	0.00	0.00	0.00	
55	♂	0.15	0.15	0.05	
23	♂	0.20	0.47	0.05	
<i>Meningitis lymphocytaria ac.</i>					
14	m	0.00	0.12	0.00	
16	f	0.20	0.10	0.00	
<i>Meningitis purulenta ac.</i>					
30	m	0.00	0.37	0.05	Leucos. 11,000
28	f	0.20	0.15	0.05	* 8,000
<i>Haemorrhagia cerebri</i>					
79	m	0.06	0.12	0.05	
66	f	0.05	0.45	0.10	

SUMMARY

The three proteolytic enzymes, pepsin, cathepsin, and trypsin, were studied from the cerebrospinal fluid in normal and various pathological conditions. In normal cerebrospinal fluids the pepsin and cathepsin showed some activity in some cases, and the trypsin was 0 in all cases. In various pathological conditions the pepsin and cathepsin showed some activity in most cases, and the trypsin showed in some cases traces of activity. No characteristic changes in various diseases could be verified.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY
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COMMENTS ON PAPER CHROMATOGRAPHY OF PHOSPHATIDES*

by

O.-V. RENKONEN and O. RENKONEN

(Received for publication January 30, 1959)

Filter paper (or glass fiber paper) impregnated with silicic acid is the best medium, available at present, for paper chromatographic differentiation of phosphatides. Several different solvent mixtures have been used as developers on these papers in studies of phosphatides (1, 2, 6—8). The most successful of these different paper chromatographic methods are those described by Marinetti *et al.* (7—8). However, it is not known exactly how the variables which are present in the impregnation technique and in the solvent mixtures of Marinetti *et al.* influence the relative mobilities of phosphatides.

This paper describes an attempt to study two such variables by comparing the paper chromatographic mobilities of monophosphoinositide and lecithin.

MATERIALS AND METHODS

Samples of Pure Phosphatides. — The monophosphoinositide (MPI), from wheat germs (3), was a gift from Dr. M. Faure. The sample was used as the sodium salt. Paper chromatographically the preparate was very homogeneous. The lecithin was synthetic L- α -dipalmitoyl-lecithin, (puriss.), obtained from Fluka AG., Buchs, Switzerland. The sample contained a small amount of a lipid similar to lysolecithin and traces of two acidic

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lipids. Two samples of sphingomyelin were used. One of them was a gift from Dr. G. V. Marinetti, and the other one was isolated from human serum (9); especially the latter seemed to be rather pure. Likewise two samples of lysolecithin, one a gift from Dr. G. V. Marinetti and the other isolated from human serum (9), were employed; both of these were probably very pure.

Chromatography on Unimpregnated Papers (11-12). — Whatman No. 1 paper was used as such. Amounts of 10—50 µg of the phosphatides were applied to the paper in 5 µl of solvent [lecithin in chloroform, sphingomyelin and lysolecithin in chloroform-methanol (1: 1),¹ and monophosphoinositide in chloroform-ethanol-water (2: 4: 1)]. The papers were left to dry in air for approximately 15—30 minutes before the chromatography was started.

The solvent systems used were:

System 1:	DBK—AcOH	(80:20)
System 2:	DBK—AcOH—H ₂ O	(80:20:3)
System 3:	DBK—AcOH	(70:30)
System 4:	DBK—AcOH—H ₂ O	(70:30:4.5)
System 5:	DBK—AcOH	(40:25)
System 6:	DBK—AcOH—H ₂ O	(40:25:5); (8)
System 7:	DBK—AcOH	(40:30)
System 8:	DBK—AcOH—H ₂ O	(40:30:7); (7)

(DBK = di-isobutyl ketone; AcOH = acetic acid)

The chromatograms were carried out at room temperature by the descending technique as described previously (10). The development time of the chromatograms was about 4 hours. In this time the solvent front moved about 20—25 cm.

The lipids were stained on chromatograms with Rhodamine 6G essentially as described by Rouser *et al.* (11).

Chromatography on Silicic Acid Impregnated Papers. — The papers (Whatman No. 1) were impregnated with silicic acid essentially as described by Marinetti and Stotz (7). For each batch of 30 paper strips (18 × 43 cm) 800 ml of fresh, diluted sodium silicate² solution was prepared. The strips were passed through this solution in 20 ± 3 seconds. The papers of batches A and B were hung, the starting line down, for 14—15 minutes before immersion into 6N HCl. The papers of batch C were hung for 0, and 5, and 10 minutes respectively. The length of the HCl-bath of individual papers varied between 20 and 90 minutes in batches A and B; in batch C the bath lasted 30 minutes. For each batch of 30 strips 10 litres of 6N HCl were used. The papers were washed two times with tap water, four times with distilled water, and hung to dry at room temperature.

The phosphatides (10—50 µg) were applied on these papers in the

¹ All solvent ratios are based on volumes.

² Sodium silicate, 40—42° Bé, obtained from E. Merck, Germany was diluted with equal volume of distilled water.

same way as on the unimpregnated papers. The solvent mixtures 5—8, listed above, were used as developers. Descending technique of chromatography was employed. The development time of the chromatograms in the systems 6 and 8 was about 16—20 hours. The solvent front moved in this time about 23—28 cm, the lecithin spot 3—11 cm. The development time in the systems 5 and 7 was approximately 20 hours. The solvent front already reached the lower end of the paper in about 15 hours. Lecithin moved 1—2 cm on papers of batches A and B, and about 4—5 cm on papers of batch C.

Staining of the lipids was carried out with Rhodamine 6 G as above. The choline containing lipids were seen as yellow spots, the MPI as a blue-brown spot under UV-light. In our hands, this staining method allowed the detection of 2—5 µg of phosphatides on actual chromatograms.

RESULTS

The R_f -values of the phosphatides on the unimpregnated papers are given in table 1.

TABLE 1
MEAN R_f -VALUES OF THE PHOSPHATIDES ON UNIMPREGNATED FILTER PAPER

Phosphatide	R_f -Values							
	System 1.	System 2.	System 3.	System 4.	System 5.	System 6.	System 7.	System 8.
MPI	0.01	0.01	0.04	0.06	0.17	0.23	—	0.45
Lecithin	0.66	0.68	0.81	0.71	0.84	0.70	—	0.80
Sphingomyelin . .	0.57	—	0.81	0.71	—	—	—	0.79
Lysolecithin . .	0.30	0.23	0.64	0.26	—	—	—	0.53

These R_f -values correspond closely to the results previously obtained by Witter *et al.* in similar systems (12). Table 1 shows that MPI is more firmly adsorbed than the choline containing lipids on cellulose. This difference, however, diminishes when the polarity of the solvent mixture is increased.

The results of the chromatograms on the silicic acid impregnated papers are given in terms of the relative mobilities of the lipids. For this purpose the ratio: distance run by a phosphatide, compared to the distance run by lecithin, is employed. This concept is called the R_f -value of the phosphatide.

Table 2 shows the R_f -values of MPI in the two Marinetti systems 6 and 8, when silicic acid papers, prepared in different ways, are used.

TABLE 2
R_I-VALUES OF MPI IN THE TWO MARINETTI-SYSTEMS (SILICIC ACID PAPERS)

Bathing Time of the Papers in HCl	R _I -Values of MPI (Lecithin = 1.00)			
	System 6		System 8	
	Batch A	Batch B	Batch A	Batch B
20—30 minutes . .	1.30	0.85	1.10	0.85
40—60 minutes . .	1.55	1.30	1.30	1.30
90 minutes . .	2.10	—	1.55	—

Table 2 shows that, other things being unchanged, the R_I-values of MPI tend to increase, when the bathing time of the papers in HCl is increased. This is mainly due to increased adsorption of lecithin on papers of long bathing times in HCl. Therefore, on papers of different bathing times, the running order of MPI and lecithin is occasionally inverted in the two Marinetti systems.

Table 3 gives, in terms of R_I-values of MPI, the comparison between the two Marinetti systems, 6 and 8, and the corresponding dry systems, 5 and 7, on several differently prepared silicic acid paper types.

TABLE 3
R_I-VALUES OF MPI IN THE MARINETTI-SYSTEMS 6 & 8 AND IN THE CORRESPONDING DRY SYSTEMS 5 & 7 (SILICIC ACID PAPERS)

Paper Type	R _I -Values of MPI (Lecithin = 1.00)											
	Batch A				Batch B				Batch C			
	System 5.	System 6.	System 7.	System 8.	System 5.	System 6.	System 7.	System 8.	System 5.	System 6.	System 7.	System 8.
1.	5.9	1.2	5.7	1.0	3.0	0.85	3.1	0.85	—	—	—	—
2.	5.8	1.6	6.6	1.3	6.0	1.3	6.9	1.3	—	—	—	—
3.	6.4	1.5	7.0	1.3	—	—	—	—	—	—	—	—
4.	5.4	2.1	9.2	1.6	—	—	—	—	—	—	—	—
5.	—	—	—	—	—	—	—	—	1.2	0.40	—	—
6.	—	—	—	—	—	—	—	—	1.6	0.31	1.2	0.38
7.	—	—	—	—	—	—	—	—	1.3	0.34	1.0	0.46

Table 3 shows that other factors being unchanged, the dry systems give 2–6 times higher R_I-values of MPI than the corresponding water containing Marinetti systems. Consequently, MPI nearly always runs faster than lecithin in the dry systems. The reason for this behavior is the very firm adsorption of lecithin on the dry silicic acid papers.

Very good separation between MPI and lecithin, on impregnated papers, is obtained, when the two factors elevating the R_f -values of MPI are combined, and chromatograms on papers of a long HCl bathing time are run with the dry systems.

Sphingomyelin and lysolecithin give R_f -values smaller than unity on all types of silicic acid papers and in all solvent systems used.

DISCUSSION

The diphosphoinositide from brain is known to be adsorbed more firmly than lecithin on cellulose (11—12). This is the case also with the MPI from wheat germs (Table 1). On the contrary, on silicic acid columns, the MPI from animal sources is known to migrate faster than lecithin, when dry developers are used (4). Therefore it is not very surprising that differences in the absolute and relative mobilities of MPI and lecithin are brought about by variations in the silicic acid impregnation of the papers. It seems that the papers of a long bathing time in HCl probably represent a purer silicic acid medium than the papers of shorter bathing times.

The great influence of water content on the relative mobilities of MPI and the choline containing lipids on silicic acid papers deserves two comments. First, the control of the drying and the storage of the papers seems to be important. Further, it seems that the dry systems 5 and 7 probably are more advantageous for paper chromatography in some cases, than the Marinetti systems 6 and 8. This is thought to be the case especially if all different phosphoinositides (5) and also the both lysocephalins would run faster than lecithin in these dry systems.

The results of this work demonstrate once again that identification of phosphatides, based solely on their R_f -values, or even on their relative mobilities on silicic acid impregnated papers are of relatively little value.

SUMMARY

The relative mobilities of monophosphoinositide and of the common choline containing phosphatides were measured on paper chromatograms. Various dry and water containing mixtures of

System
8.

0.38
0.46

di-isobutyl ketone and acetic acid were used as developers on unimpregnated papers, and on papers differently impregnated with silicic acid. Two variables influencing the relative mobilities of these lipids on the silicic acid papers were demonstrated.

Acknowledgement. — We wish to thank also here Dr. M. Faure, Paris, for her generous gift of wheat germ phosphoinositide. We also wish to thank Dr. G. V. Marinetti, Rochester, USA, for his help and his gifts of sphingomyelin and lysolecithin.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY
OF HELSINKI

PAPER CHROMATOGRAPHY OF NONPHOSPHOLIPIDS IN
SOME SYSTEMS DEVISED FOR ANALYSIS
OF PHOSPHATIDES¹

by

O.-V. RENKONEN and O. RENKONEN

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Methods for paper chromatographic analysis of nonphospholipids contaminating the different phosphatide fractions are urgently needed in many phases of preparative isolation and fractionation of phosphatides. However, only a limited amount of useful information is available on the paper chromatographic behaviour of both the phosphatides and the nonphospholipids in the same systems. Marinetti *et al.* (6) described the behaviour of some nonphospholipids in the principal phosphatide system devised by themselves. They also developed a system for differentiation of some lipophilic nonphospholipids (7, 8). The behaviour of some phosphatides in this system was also described by them. — However, their systems do not adequately cover the whole polarity range needed for phosphatide work. Also, the number of nonphospholipids tested in their systems is relatively small.

This paper describes the paper chromatographic behaviour of some common nonphospholipids in the main phosphatide system of Marinetti *et al.* (6), as well as in some phosphatide systems developed in this laboratory (9, 10). The differentiation of some lipophilic nonphospholipids from each other is also described. For this purpose the nonphospholipid system of Marinetti *et al.* (7, 8) and some related systems were used.

¹ This work was supported in part by a grant from University of Helsinki.

MATERIALS AND METHODS

Samples of Pure Lipids. — The origin of the pure lipids used in this work is given in Table 1.

Paper Chromatography. — Silicic acid impregnated filter papers (4) were used. The papers were prepared as described earlier (9), except that the impregnated papers were hung for 10 ± 2 minutes before immersion into HCl. The papers were bathed in HCl for 34 ± 4 minutes. The lipids were applied on the papers and the chromatograms were developed and stained with Rhodamine 6 G (11) as described earlier (9).

The Rhodamine staining method easily allows the detection of approximately 5—10 µg of pure cholesterol esters, triglycerides, sterols, diglycerides, phosphatides, taurocholic acid and gangliosides. For compounds containing only one fatty acid radical and for free bile acids this staining method is not quite as sensitive. The blue-brown colours of the anionic lipids (fatty acids¹, cardiolipin, phosphatidyl serine², taurocholic acid and ganglioside) are in our experience much easier to detect than the yellowish colours of the other lipids.³ Some blurring of the more water soluble taurocholic acid and ganglioside spots occurs now and then during the staining.

The following solvent mixtures were used as developer systems:

1. n-heptane-DBK (96: 6); (7)
 2. n-heptane-DBK (1: 1)
 3. DBK
 4. DBK-AcOH (90: 10)
 5. DBK-AcOH (40: 25); (9)
 6. DBK-AcOH-H₂O (40: 25: 5);(6)
- (DBK = di-isobutyl ketone; AcOH = acetic acid)

The chromatograms were developed until the solvent front had moved about 20—25 cm. This was achieved in system 1 in about 4—5 hours, in systems 2, 3 and 4 in about 7—9 hours, in system 5 in about 12 hours, and in system 6 in about 16—18 hours.

RESULTS

No adequately measurable differences were observed between the mobilities of lipids within single lipid classes of table 1.⁴

¹ Fatty acids show blue colours on chromatograms developed with neutral solvents, but they give yellow colours on chromatograms developed with acidic solvents.

² The blue-brown colour of phosphatidyl serine turns into brown and yellow during the inspection of chromatograms under UV-light.

³ Ergosterol gives a bright blue colour. Desoxycholic acid and cholic acid give greenish-yellow colours.

⁴ Within the "classes" of bile acids and cerebrocides there are, quite naturally, differences between the different compounds. Ergosterol probably also runs a little slower than other sterols.

TABLE I
ORIGIN OF THE USED LIPID SAMPLES

Lipid Class	Compound	Origin
Cholesterol esters	Cholesterol stearate Cholesterol oleate	The British Drug Houses Ltd., Poole, England The British Drug Houses Ltd., Poole, England
Triglycerides	Trimyristin Tripalmitin Tristearin Trilinolein	G. A. F. Kahlbaum, Berlin The British Drug Houses Ltd., Poole, England E. Merck AG, Darmstadt, Germany The Hormel Institute, Austin, Minnesota
Fatty acids	Palmitic acid Oleic acid	E. Merck AG, Darmstadt, Germany E. Merck AG, Darmstadt, Germany
Sterols	Cholesterol Sitosterol Stigmastanol Ergosterol	The Coleman & Bell Co., Norwood, Ohio S. A. F. Hoffmann-La Roche & Co, Basle, Switzerland S. A. F. Hoffmann-La Roche & Co, Basle, Switzerland S. A. F. Hoffmann-La Roche & Co, Basle, Switzerland
Diglycerides	1,3-Dipalmitin	Dr O. S. Privett, Austin, Minnesota
Monoglycerides	1-Monopalmitin 1-Mono-olein	Eastman Organic Chemicals, Distillation Products Industries, Rochester, New York
Bile acids	Lithocholic acid Desoxycholic acid Cholic acid Na-Taurocholic acid	Mann Fine Chemicals, Inc., New York S. A. F. Hoffmann-La Roche & Co, Basle, Switzerland S. A. F. Hoffmann-La Roche & Co, Basle, Switzerland E. Merck AG, Darmstadt, Germany
N-(2-hydroxyethyl)-amides	Palmitic acid derivative (1)	Dr. J. Hukki, Helsinki
Cerebrosides	Galakto-cerebroside Gluko-cerebroside Disacharide-cerebroside	Prof. E. Klenk, Köln Prof. E. Klenk, Köln Prof. E. Klenk, Köln
Gangliosides	Brain ganglioside	Prof. E. Klenk, Köln
Polyglycerophosphatides	Cardiolipin	Dr. M. Faure, Paris
Phosphatidyl serines	Brain serine-cephalin	Prof. E. Klenk, Köln
Lecithins	Dipalmitoyl-L- α -lecithin Disteroyl-L- α -lecithin	Fluka AG, Buchs, Switzerland Dr. G. V. Marinetti, Rochester, New York
Lyssolethins	Serum lyssolethithin Stearoyl-L- α -lyssolethithin	Own preparation (10) Dr. G. V. Marinetti, Rochester, New York

Therefore the results can be presented in terms of R_f -values of the whole lipid classes.

Table 2 shows some typical R_f -values of both the nonphospholipids and the phosphatides in three useful phosphatide systems.

TABLE 2
TYPICAL R_f VALUES¹ OF NONPHOSPHOLIPIDS AND OF SOME PHOSPHATIDES IN THE THREE SYSTEMS, 4—6, DEVISED FOR PHOSPHATIDES

Lipid	Typical R_f Values		
	System 4	System 5	System 6
Cholesterol esters	>0.80	>0.80	>0.80
Triglycerides	>0.80	>0.80	>0.80
Fatty acids	>0.80	>0.80	>0.80
Free sterols	>0.80	>0.80	>0.80
1,3-Dipalmitin	>0.80	>0.80	>0.80
Lithocholic acid	0.70	>0.80	>0.80
Monoglycerides	0.52	0.75	0.75
Front edge of cardiolipin	0.30	0.90	0.86
Palmitic acid ethanolamide	0.24	0.69	0.70
Desoxycholic acid	0.21	0.65	0.65
Galacto-cerebroside	—	—	0.61 ²
Glucocerebroside	0.01	0.60 ²	0.66 ²
Disaccharide-cerebroside	—	—	0.43 ²
Cholic acid	0.04	0.46	0.60
Phosphatidyl serine	0.03	0.35	0.55
Lecithin	0.00	0.03	0.35
Lysolecithin	0.00	0.00	0.11
Na-Taurocholic acid	0.00	—	0.08 ³
Ganglioside	—	—	0.005

¹ The R_f -values tend to vary a little in different runs. The given figures, therefore, can only roughly indicate the mobilities of the lipids.

² Streak to $R_f = 0.00$.

³ Two spots; $R_f = 0.08$ and 0.02.

Table 2 shows that many unpolar nonphospholipids move in system 4 faster than cardiolipin, which is one of the fastest moving phosphatides. This fast moving group of the nonphospholipids includes cholesterol esters, triglycerides, fatty acids, free sterols, diglycerides, lithocholic acid and monoglycerides. — Table 2 also shows that in systems 5 and 6 cardiolipin already runs as fast as the fastest ones of the nonphospholipids.

The lipids which in system 4 run with the same rate as, or slower than cardiolipin include desoxycholic and cholic acids, palmitic acid ethanolamide, cerebrosides, taurocholic acid and brain ganglioside. Of these lipids only the ganglioside runs clearly behind the phosphatide region. The mobilities of all the others are within the phosphatide range in all three systems.

For analysis of the unpolar nonphospholipids the systems 1—3 are suitable. Typical R_f -values for these systems are given in Table 3.

TABLE 3.
TYPICAL R_f -VALUES¹ OF THE UNPOLAR NONPHOSPHOLIPIDS AND OF SOME PHOSPHATIDES IN THE THREE SYSTEMS, 1—3

Lipid	Typical R_f -Values		
	System 1	System 2	System 3
Cholesterol esters	0.82	0.86	0.87
Triglycerides	0.66	0.84	0.87
Fatty acids	0.48	0.75	0.80
Free sterols	0.25	0.75	0.81
Dipalmitin	0.09	0.75	0.79
Lithocholic acid	0.01	0.22	0.53
Monoglycerides	0.01	0.13	0.30
Front edge of cardiolipin	0.00	0.04	0.09
Palmitic acid ethanolamide ..	0.00	0.03	0.05
Desoxycholic acid	0.00	0.01	0.04
Lecithin	0.00	0.00	0.00
Lysolecithin	0.00	0.00	0.00

¹ The R_f -values tend to vary a little in different runs. The given figures, therefore, can only roughly indicate the mobilities of the lipids.

DISCUSSION

Our results, as well as those reported by Hokin and Hokin (3), show that clear separations between the unpolar nonphospholipids and the rapidly moving phosphatidic acids are not easy to obtain in the principal phosphatide system of Marinetti *et al* (6). For this purpose our system 4 seems to be more suitable.

On the basis of our results it seems possible that many free and conjugated bile acids and also many cerebrosides might seriously interfere with paper chromatographic analysis of phosphatides in

systems described by Marinetti *et al.* (5, 6) and also in systems of this laboratory (9).

The series of solvent systems presented in this work is suitable for analysis of lipid fractions obtained with silicic acid column chromatography of serum lipids (10, 2).

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SUMMARY

Chromatography of some naturally occurring nonphospholipids on silicic acid impregnated papers is described.

Special attention is paid to separations between various unpolar nonphospholipids, and to their differentiation from the most rapidly running phosphatides.

Some bile acids and cerebrosides are shown to have mobilities in the phosphatide range in the used systems.

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PRECIPITATION OF SERUM PROTEINS WITH SULFO-SALICYLIC ACID

by

S. MATTILA and K. O. RENKONEN

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The turbidity of sera precipitated with sulfosalicylic acid seems to increase in young age (1). The purpose of this work has been to secure this observation and simultaneously to study this precipitation in some pathological conditions. The amount of serum proteins was estimated for comparison as well as the sedimentation rate. In few cases the thymol test was also performed.

MATERIAL

The blood was collected at the morning from patients of the First and Second Medical Clinics of the Central University Hospital in Helsinki.

METHODS

Before the precipitation the sera were diluted with saline to 1:200. Equal amounts of this dilution and the sulfosalicylic acid (20% in water) were mixed in a test tube. The extinction was measured after 15 minutes at room temperature with a Zeiss spectrophotometer (PMQ II) using the wavelength of 650 m μ and the length of cells was 10 mm. The amount of serum proteins was estimated by the Folin Ciocalteau method. The thymol test was performed as usually.

RESULTS

The results are collected in the following table, where they are divided in seven groups on the basis of diagnoses and also in two healthy groups.

TABLE I

Group	Sulfosalicylic Acid Precipitation Extinction		S. R. mm/h		Amount of Protein Per Cent		Age		Examiners
	Mean	Distribution	Mean	Distribution	Mean	Distribution	Mean	Distribution	
Malignant tumors	0.280	0.195—0.385	44	3—108	7.7	5.8—9.6	58	33—77	21
Nephritis chronic	0.300	0.205—0.365	59	3—128	7.2	6.0—8.3	44	22—66	10
Hypertension ess. . . .	0.350	0.210—0.515	31	1—97	7.3	6.0—9.4	61	36—81	24
Arteriosclerosis	0.370	0.240—0.550	24	2—64	7.2	6.1—8.9	63	41—76	23
Diabetes mellitus	0.380	0.240—0.630	20	2—62	7.1	6.0—8.7	48	18—76	17
Tuberculosis	0.390	0.235—0.555	56	4—160	6.8	5.9—7.9	40	18—68	12
Acute infections	0.400	0.205—0.660	38	2—130	7.3	6.0—8.8	40	15—79	38
Healthy people (old) . .	0.415	0.275—0.690	12	2—28	7.4	6.0—8.9	55	44—82	22
" " (young)	0.480	0.320—0.720	10	2—27	7.5	6.5—8.8	24	15—34	22

DISCUSSION

It is evident that the sera taken from healthy people precipitated more strongly with sulfosalicylic acid than the sera from sick people. The mean turbidity extinction with sulfosalicylic acid in the group of healthy young people was almost twice as high as the mean in the group of malignant tumors. The distribution of extinction values was analogous.

The turbidity seemed to be especially low in malignant tumors and in chronic nephritis. In the latter group the sedimentation rates (mean 59 mm) were lower than in the former (mean 44 mm). Thus the pattern of serum proteins in these groups must be of different kind. The low extinctions in the group of malignant tumors could be due to mucoproteins, which are increased in malignant tumors independent of localisation and type (2, 5). The mucoproteins are rather inhibiting the precipitation reactions (3). Also in the other groups mucoproteins may play some role. According to different investigations (4, 5) mucoproteins are in almost every pathological condition increased,

although overlapping values occur in normal state. On the other hand the amount of mucoproteins increase in old age (4, 5). Apparent the precipitation phenomenon is very complex. Many factors and interactions might be involved.

The turbidity with sulfosalicylic acid did not seem to depend on the severity of disease only, but also on the age. So e.g. in tuberculosis where the somewhat high sedimentation rate indicated a rather severe pathological condition, the mean extinction was, however, stronger than in hypertension, where the sedimentation rate was a half of that in tuberculosis. The explanation here can be partly due to the difference between the mean ages, which is in tuberculosis forty years and in hypertension group 61 years.

The total amount of serum proteins varied but little in different groups. Thus the differences in the precipitation reaction can't be explained that way.

With some sera the thymol test was also performed. The results were as follows:

Thymol Test	Extinctions ¹ with Sulfos. Acid Mean	Number of Sera
>2.0	390	185—530 22
<2.0	395	195—570 26

No correlation could be noted.

SUMMARY

The precipitation of serum proteins with sulfosalicylic acid was investigated and the precipitation was found to be depended on the age and disease of the patient.

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¹ These extinctions were measured using a Bausch et Lomb spectrophotometer.

FROM THE DEPARTMENT OF PATHOLOGY, UNIVERSITY OF HELSINKI

TUMOR-PROMOTING EFFECTS IN MOUSE SKIN

COMPARISON BETWEEN OLEIC ACID, SORBITAN MONO-OLEATE (SPAN 80),
AND POLYOXYETHYLENE SORBITAN MONO-OLEATE (TWEEN 80)

by

PAUL HOLSTI

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Following the establishment of the tumor promoting effect of the non-ionic lipophilic-hydrophilic Tweens and Spans (4, 6), and the experiments carried out with these compounds on the mechanism of tumor promotion (1, 3, 5, 7, 8), it was shown that some of the long chain fatty acids contained in the molecule of these compounds were able to exhibit tumor promoting properties in the mouse skin even when used as free acids (2). A comparison of the promoting activity of a Tween, a Span and their respective fatty acid was therefore undertaken under as identical conditions as possible. Because oleic acid offers special advantages, being a liquid, over the other fatty acids of the various Tweens and Spans, this acid and a Tween and a Span containing it were chosen as subjects of the investigation. The purpose of the experiment was exclusively to compare the tumor promoting effect of the chosen compounds when applied on a previously initiated area of mouse skin. For the effects of these compounds when used without foregoing initiation see ref. 2 and 4.

MATERIAL AND METHODS

The experimental animals were male and female mice of an anonymous outbred strain, raised (and temporarily in use) in this

Research Assistant in Pathology, by Aid of Rockefeller Foundation Grant.

laboratory, and not identical with the strain used in the previous experiments with Tweens and Spans (4, 6). The animals were 4 to 6 months old at the commencement of the experiment and with respect to age and sex exactly equally distributed among the various groups, each of which comprised 44 animals (22 male and 22 female mice). The same stock diet as in the previous experiments (2, 4) was used and water was given ad libitum.

Initiation was accomplished in all the series on the same day and by the same person, by a single, local application of a 0.3 per cent solution of 9,10-dimethyl-1,2-benzanthracene (DMBA) in colorless, non-fluorescent liquid paraffin. The investigated compounds were: Tween 80 (polyoxyethylene sorbitan monooleate) and Span 80 (sorbitan monooleate) from Atlas Powder Co., USA, and oleic acid from May & Baker Ltd., England. The treatment with these compounds was started 24 hours after the initiation and the compounds were applied with a No. 2 water color brush by the same person once daily (six times a week) to the previously initiated area of skin on the animals back. The tumors were recorded once weekly and the incidence of tumors was calculated from the number of animals surviving at the time of appearance of the first tumor in the series concerned.

RESULTS

The development of skin tumors in the various groups is pictured in Fig. 1, while the number of tumors and of surviving animals are listed in Table 1. The experiment lasted 24 weeks.

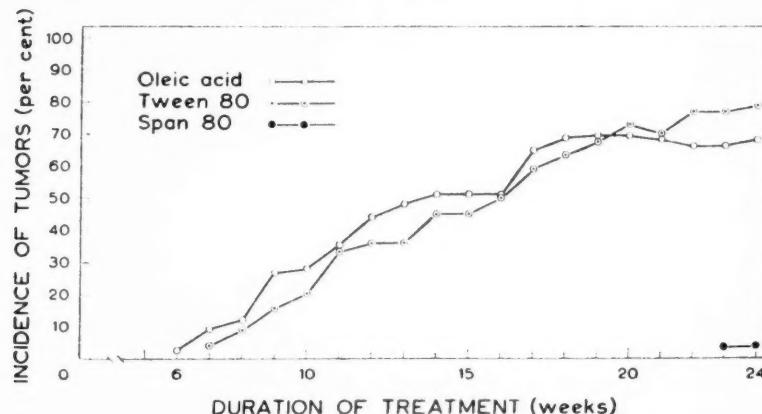


Fig. 1.—Development of local skin tumors with daily (6 times a week) treatment with oleic acid, Tween 80 and Span 80 after initiation with a single local application of 0.3 per cent DMBA in liquid paraffin.

TABLE 1

Test Compound	First Tumor		Maximum Incidence of Tumors		No. of Mice Alive at 24 Weeks
	Time (Weeks)	No. of Mice Alive	Time (Weeks)	Tumors per Tumor-Bearing Mouse	
Oleic acid	6	43	19	131/30	36
Tween 80	7	44	21	143/35	43
Span 80	23	38	23	1/1	38

As seen from Fig. 1, the incidence of skin tumors in the series treated with oleic acid developed in a manner closely similar to that in the series treated with Tween 80.

The number of tumors per tumor bearing animal (Table 1) in these two series also largely corresponds, while in the series treated with Span 80 only one tumor was observed.

DISCUSSION

Most of the fatty acids contained in the molecule of the various Tweens and Spans (stearic, palmitic and lauric acids) are solid and practically insoluble in water. Therefore a direct comparison of the effects on the skin of these acids and their respective Tween- and Span-type products is not feasible. In this respect oleic acid, being a liquid, is an exception and makes such a comparison possible. When, in the present work, undiluted oleic acid and polyoxyethylene sorbitan monooleate (Tween 80) were used as promoters under identical experimental conditions, tumors developed according to a closely similar pattern in the two series. It should be borne in mind, however, that the molecule of Tween 80 contains only about 21.5 per cent oleic acid (4). The incorporation of oleic acid into the total molecule of Tween 80 probably makes it more soluble and more readily transportable. This does not exclude the possibility that the total molecule of the Tweens may still have other important properties that influence the development of tumors. It is also to be noted that Span 80 (sorbitan monooleate) in the present investigation, as in the previous experiments (4), exhibits only feeble tumor promoting activity. The actual incidence of tumors in the

present work should not be compared with that in the earlier investigation with the Tweens and Spans (4), since a different strain of mice was used.

SUMMARY

The tumor promoting activity in the mouse skin of oleic acid, sorbitan mono-oleate (Span 80) and polyoxyethylene sorbitan monooleate (Tween 80) was compared.

Under the conditions of the present experiment and during the period of observation of 24 weeks the development of local skin tumors followed a practically identical course in the series treated with oleic acid and that treated with Tween 80. In agreement with the results of previous investigations, Span 80 exhibited only very weak tumor promoting activity.

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FROM THE DEPARTMENT OF PATHOLOGY, UNIVERSITY OF HELSINKI

HYPERPLASTIC EFFECT OF FATTY ACIDS AND ALCOHOLS OF VARIOUS CHAIN LENGTHS ON MOUSE SKIN

by

PAUL HOLSTI

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Certain technical (5, 7), as well as laboratory-synthesised (6) non-ionic surface active compounds which are polyoxyethylene sorbitan esters or sorbitan esters of long chain fatty acids (*e.g.*, some of the Tweens and Spans), exhibit, when used according to the frequent-application technique (5), potent tumor promoting effects in the mouse skin. The degree of epidermal hyperplasia elicited by these compounds is positively correlated to the tumor promoting effect of the respective compound and to the frequency of treatment (2, 4). Some of the long chain fatty acids contained in the molecule of the above mentioned surface active compounds were then shown to exhibit tumor promoting properties also when used as free acids (3).

In order to investigate the significance of the length of the carbon chain for the elicitation of this effect, fatty acids and alcohols of various chain lengths (with an even number of carbon atoms) were tested for a possible hyperplastic effect on the epidermis of the mouse skin. The experiment was pursued partly also in order to seek suitable concentrations of the various fatty acids for tumor promotion experiments.

The experimental animals, totaling 1600, were white male and female stock mice (3). The following compounds were investigated: Linoleic acid, C 18 (Fluka AG, Switzerland); oleic acid, C 18 (May & Baker, Ltd., England); stearic acid, C 18 (E. Merck AG, Germany);

palmitic, myristic and lauric acid, C 16—12) (Eastman Kodak Co., U.S.); caprinic, caprylic, caproic, butyric and acetic acid, C 10-C 2 (Fluka AG); oleyl alcohol and decyl alcohol (Fluka AG); capryl alcohol (E. Merck AG); hexyl alcohol (Fluka AG); butyl alcohol (May & Baker) and ethyl alcohol (*absolutus*). They were used undiluted when possible, and in various concentrations dissolved in chloroform.

The compounds were applied once daily to an area of the back skin, which was clipped free of hair with scissors. On specified days five mice from each series were killed, the treated area of the skin was excised and fixed in Susa mixture, and sections prepared according to the previously described method (2). In addition to the usual histological evaluation of the alterations, a histo-quantitative analysis (method in ref. 2) was performed in cases suitable for such an analysis.

The results show that the ability to induce a hyperplastic reaction in the epidermis is not restricted only to the previously investigated fatty acids (3) contained in the molecule of some of the non-ionic surface active tumor promoters. Of the higher fatty acids which, because of their liquid constitution, could be used undiluted, linoleic and oleic acids (unsaturated C 18) exhibited significant hyperplastic properties, as has been observed also elsewhere (1). The next shorter liquid fatty acid, caprylic acid (saturated C 8), still clearly induced epidermal hyperplasia, being, however, in addition more destructive. When proceeding in the fatty acid series towards the shorter ones, this property of inducing strong destructive alterations in the skin became increasingly prominent. This precluded the use of higher concentrations of the lower fatty acids and tended to obscure how far in the fatty acid series the ability to induce epidermal hyperplasia extends.

For this reason, similar experiments were undertaken with a series of alcohols corresponding in chain length to some of the used fatty acids. The lower alcohols did not cause any destructive alterations in the skin, in contrast to the corresponding fatty acids. This made a comparison between undiluted compounds of various chain lengths possible. The use of the alcohols for this purpose was justified by the observation, made in this laboratory, that not only oleic acid but also the corresponding alcohol, oleyl alcohol, was able to induce the hyperplastic reaction in the epidermis.

Table 1 gives an example of the hyperplastic effect of the tested alcohols and of linoleic and oleic acids, as pictured by the epidermal cell count on the seventh day of treatment. All animals in this series were female mice.

TABLE 1

CELL COUNTS* IN MOUSE EPIDERMIS AFTER SIX DAILY APPLICATIONS OF THE LISTED COMPOUNDS

Compound	Basal cells	Differ. cells	Degen. cells	Mitotic cells	Total cells
Linoleic acid	177	148	9	1.6	335
Oleic acid	180	142	4	2.6	329
Oleyl alcohol	189	172	15	2.4	378
Decyl alcohol	153	133	12	2.2	300
Capryl alcohol	166	169	6	0.8	322
Hexyl alcohol	150	96	6	2	254
Butyl alcohol	168	101	7	1.2	277
Ethyl alcohol	169	74	3	0.6	246
None	170	47	8	1.2	226

* See ref. 2

When viewing the results of the alcohol series as a whole it appears that the hyperplastic effect in general tends to decrease with the decreasing carbon chain lengths of the alcohols, although exceptions may occur. Judged by the microscopic appearance of the skin, the differences in the hyperplastic effect were even greater than would appear from the cell counts, because of the increased size of individual cells and the exclusion of the str. granulosum from the cell count (see method in ref. 2). The lower alcohols, ethyl alcohol, butyl alcohol and hexyl alcohol thus had only a very small effect, the hyperplastic effect practically disappearing in the area between capryl alcohol (C 8) and hexyl alcohol (C 6). The results of closer comparisons of especially the fatty acids in this respect, as well as the results of pertinent tumor promotion experiments will be reported later.

SUMMARY

A series of fatty acids and some of the corresponding alcohols were tested on mouse skin in order to study the significance of the carbon chain length of the compound for the elicitation of the epidermal hyperplastic effect.

The liquid fatty acids linoleic, oleic and caprylic acid, which could be used undiluted, all exhibited a clear hyperplastic effect. The destructive effects, exhibited especially by the lower fatty acids, precluded the use of these acids in higher concentrations and rendered the evaluation of their effects difficult.

In the alcohol series the intensity of the hyperplastic effect tended in general to decrease with decreasing carbon chain lengths, the effect practically disappearing in the area between capryl alcohol (C 8) and hexyl alcohol (C 6).

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FROM THE DEPARTMENT OF PATHOLOGY, UNIVERSITY OF HELSINKI

AMYLOID-LIKE INFILTRATION IN MICE OF A NON-INBRED STRAIN

WITH REFERENCE TO ITS RELATION TO TREATMENT WITH CERTAIN
SURFACE ACTIVE COMPOUNDS

by

PAUL HOLSTI

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In aging mice of inbred strains, amyloid disease of various internal organs is a frequent finding (2, 3, 4, 8, 9, 10).

During the course of experiments in skin carcinogenesis where certain surface active compounds were applied to the skin of mice of a non-inbred strain (1, 5, 7), hyaline alterations were noted in the internal organs of several treated mice as well as in some of the control animals. The present paper gives a description of these alterations and discusses them against the background of some previously known forms of hyaline disease in mice.

MATERIAL AND METHODS

Specimens were taken from altogether 700 male mice of an anonymous non-inbred strain (7), which had been treated with various non-ionic surface active compounds (*e.g.*, Tweens and Spans) and related substances (7), and from 130 control mice of the same strain. The investigated compounds had been applied to an area (2×1.5 cm) of skin on the animals' back once or twice daily (six or twelve times a week). The mice were 2 to 3 months old at the commencement of the treatment. A smaller part of the material came from biopsy series of up to 3 months' duration,

while most of the specimens were from tumor promotion series of up to 52 weeks' duration. In these series only random samples had been taken from each series, as no special alterations were anticipated at the time in the organs. Ten per cent aqueous formalin was used for fixation. The material was embedded in paraffin and the routine sections were stained by the hematoxylin-van Gieson method (HvG). Other stains used appear from the text.

RESULTS

Incidence of Alterations

Because most of the specimens were taken as random samples from a few animals in each tumor promotion series, a statistical comparison of these series is not feasible. Table 1 shows the results of a comparison between the untreated control mice (age 6 to 9 months) and the older mice (age 5 to 6 months) from the biopsy series in which liver and kidney specimens were taken from every animal.

TABLE 1

INCIDENCE OF UNTREATED MICE AND MICE TREATED WITH NON-IONIC SURFACE ACTIVE COMPOUNDS SHOWING HYALINE ALTERATIONS. DURATION OF TREATMENT FROM 2 TO 3 MONTHS

	Untreated	Treated
Liver	3/130 (2 per cent)	16/122 (13 per cent)
Kidney	0/130	5/122 (4 per cent)

The kidney changes were found in mice treated with Tween 60 (polyoxyethylene sorbitan monostearate), Tween 80 (polyoxyethylene sorbitan monooleate) and Tween 40 (polyoxyethylene sorbitan monopalmitate).

Liver alterations were observed in series treated with Tween 60, Tween 80, Tween 81 (polyoxyethylene sorbitan monooleate), Tween 20 (polyoxyethylene sorbitan monolaurate), Span 85 (sorbitan trioleate), Span 80 (sorbitan monooleate) and G-2854 (polyoxyethylene sorbitol tetraoleate). In some of the animals, treated as well as untreated, hyaline alterations were seen also in the spleen, and in one treated animal in the adrenal. Specimens from these organs, however, had only been taken occasionally.

Character of Alterations

In the liver the optically homogeneous, slightly eosinophilic material was found deposited along the hepatic sinusoids (Fig. 1).

The adjacent parenchymal cells were atrophic or hyalinized. The deposited material stained only lightly with Congo red but differed from its surroundings by taking on a more violet color with methyl-violet and gentian-violet, without, however, giving a clear metachromatic reaction. Staining with dilute Lugol solution did not differentiate the material from the surrounding tissue. When sulphuric acid was added after treatment with Lugol solution a transient violet color was noted.

In the spleen the hyaline material occurred mainly in the peri-follicular area, often forming incomplete rings around the follicles (Fig. 2).

The staining qualities of this material were similar to those of the hyaline material in the liver.

The alterations in the adrenals occurred mainly in the peri-medullary zone and extended well into the cortex. The medulla was usually not affected.

Changes in the kidneys were noted only in treated animals. These alterations consisted usually of hyalinization of the glomerular loops without involvement of the afferent arterioles (Fig. 3). In addition, dilatation of the tubules with cast formation, and a slight accumulation of hyaline material in the interstitial tissue in the renal papilla were observed (Fig. 4).

The casts were usually homogeneous, yellow (HvG) and benzidine-negative and did not react with Gmelin's stain for bile pigment. In no case was necrosis of the papillary tip seen.

DISCUSSION

The alterations observed in the livers, spleens and adrenals of the experimental animals (both treated and untreated) appear to correspond in optical appearance and staining qualities to the changes described in aging mice of inbred strains (8, 9). These changes have been variously characterized by different authors and labelled as either hyaline or, despite the variable staining reactions, as amyloid. It is now generally felt that several varieties

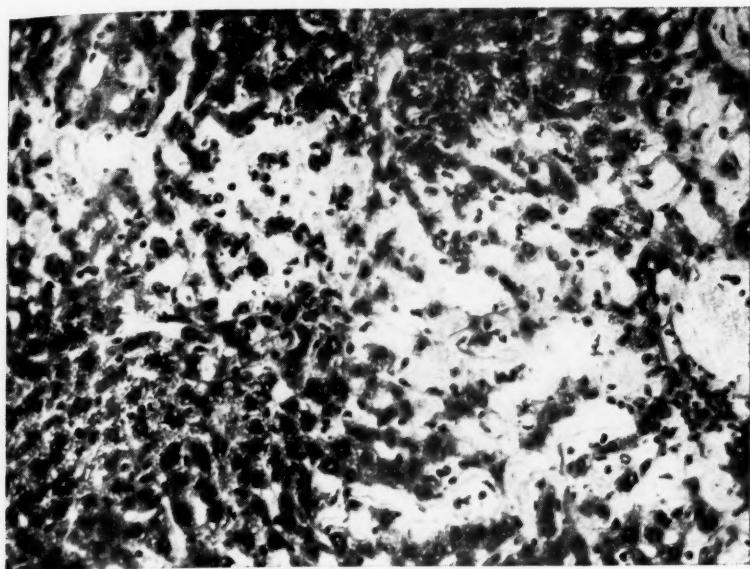


Fig. 1.— Hyaline alterations in the liver of a 6 month old mouse. Hematoxylin-van Gieson. 150/1.



Fig. 2.— Mouse No. 2087, treated twice daily with the non-ionic detergent Span 20. Predominantly perifollicular hyaline deposits in the spleen. Hematoxylin-van Gieson. 35/1.

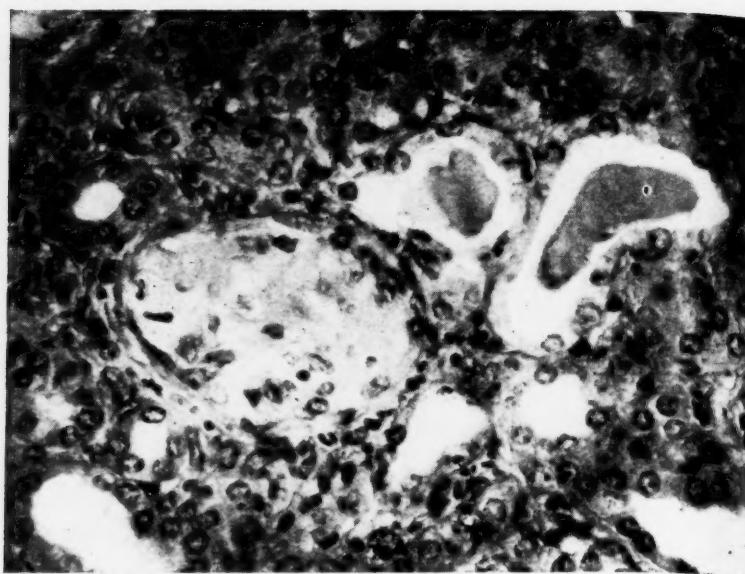


Fig. 3. — Mouse No. 2163, treated twice daily with the non-ionic detergent Tween 60. Hyalinization of glomerular loops and alterations in the tubules. Hematoxylin-van Gieson. 340/1.

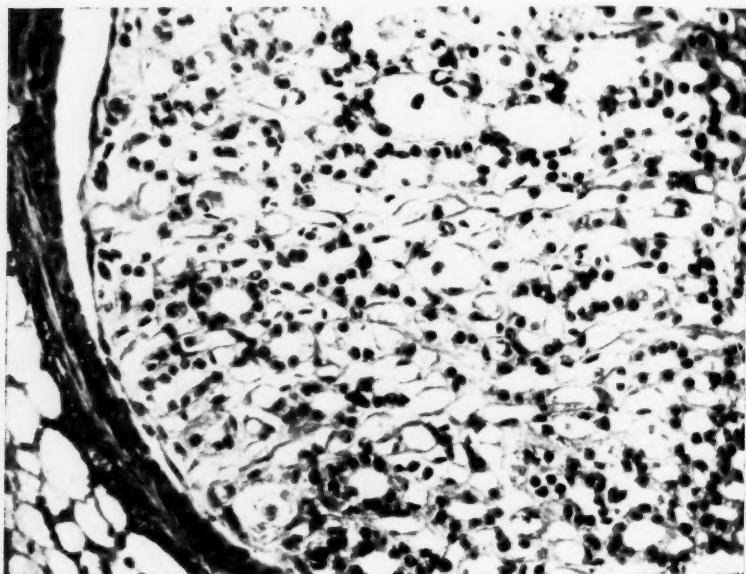


Fig. 4. — Mouse No. 2192, treated twice daily with the non-ionic detergent Tween 80. Slight hyalinization of the renal papilla. Hematoxylin-van Gieson. 155/1.

of amyloid substance exist and the specific staining reactions are not always considered of decisive importance for the designation of a hyaline substance as amyloid. The results of the specific staining procedures, furthermore, vary with the method of fixation, formaline in certain cases preventing the reaction with iodine and iodine-sulphuric acid stains. (For a review of these subjects see ref. 8).

The findings of the present investigation show that the possibility of a spontaneous hyalinosis, corresponding in appearance to senile amyloidosis, must be taken into account also in fairly young mice of non-inbred strains.

The complete absence of glomerular alterations in the control mice as contrasted to the conspicuous hyalinization of the glomeruli in the mice treated with the surface active compounds focuses attention on the possibility that this treatment may have contributed to the induction or localization of the hyaline alterations. In experiments pursued in this laboratory using ionic and non-ionic surface active compounds as solvents for carcinogen, alterations in the internal organs have also frequently developed (6). It is well known that hyaline (and amyloid) alterations are readily induced in mice by a wide range of procedures (8). The non-ionic surface active compounds used in the present experiments are, according to investigations pursued also in this laboratory, fairly inactive chemically. While they cause a slight alteration in the electrophoretic behavior of the globulins, their principal action is apparently linked to their physicochemical properties (7). Both these facts may have a certain bearing on the problem of amyloid formation, which increasingly is associated with disturbances in colloidal stability and antigen-antibody relations (8).

SUMMARY

Specimens from various internal organs of 700 mice treated with surface active compounds as well as of 130 control mice of the same non-inbred strain were examined.

Hyaline, amyloid-like infiltrations, corresponding in appearance to so called senile amyloidosis of mice, were observed in the liver and spleen of both treated and untreated animals. The results show that the possibility of a spontaneous hyalinosis has to be

taken into account also in fairly young mice (aged 5 to 6 months) of non-inbred strains.

Alterations in the kidneys, especially hyalinization of the glomerular loops, was seen only in animals treated with certain surface active compounds. The implication of this finding and its relation to problems of amyloid formation are discussed.

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FROM THE INSTITUTE OF PHYSIOLOGY, UNIVERSITY OF HELSINKI

EFFECT OF CAMPHOR UPON THE COAGULATION OF BLOOD

by

ANNIKKI KIVALO

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Camphor has always been used as an analeptic and in pulmonary haemorrhages as an haemostypnic. It has to be admitted, though, that the efficiency of its activity in the first-mentioned sense is controversial nowadays. The haemostypnic effect of camphor in haemorrhage of the lungs has been attributed to the circumstance that, by increasing the pressure in the greater circulation, it reduces the pressure in the lesser circulation. However, in the case of occurrence of pulmonary haemorrhage the pressure in the lesser circulation must not necessarily be elevated and the effect of camphor on normal circulation is nowadays considered as non existent. For this reason the question of the mechanism of effect of camphor was brought up once more, primarily its *effect upon the mechanism of blood coagulation* being concerned (7).

Several substances promoting the coagulation of blood are known (4, 13). *Directly acting*, also *in vitro*, are substances containing some component concerned in the mechanism of coagulation (blood, thrombocytes, their extracts, tissue fluids). An *indirect effect* occurs with agents mobilizing in one way or another substances of the above-mentioned kind, e.g. by breaking down thrombocytes (water, alcohol, ether). The effect manifests itself *in vivo* only if, e.g., the substance in question, by causing increased hydraemia, introduces coagulation components in the circulation as do digitalis, strophantin and mercurial diuretics in cardiac insufficiency patients (8). An coagulation promoting effect is

noted as a rule with concentrated salt and sugar solutions (3, 12). Of the so-called convulsive poisons, to which also camphor is considered to belong, picrotoxin and strychnine have an effect on the coagulation of blood. *In vitro*, these substances only work in high concentrations, and then, they act as inhibitors (11). The results stated in literature are frequently contradictory. For instance, Secher (10) noted that digitalis shortens also *in vitro* the time of coagulation, which fact Pere (8) could not confirm in his material. — The nervous system, has likewise an effect on coagulation in that sympathetic stimulation promotes coagulation (13). Furthermore, factors associated with hormones also enter the picture.

MATERIAL AND METHODS

The experiments were performed on nine healthy subjects between 21—37 years of age. The time of coagulation was determined according to Bürker's method (1), particular attention being paid to uniform temperatures in the apparatus (25°C) and in the room (19°C), to the oozing out of the drop from the finger without any pressure being applied, and to the purity of the drop of water. Freedom of dust in the laboratory air was found to be of primary importance for the reason that small dust particles easily acted as so-called centres of coagulation, thus promoting the coagulation. Blood was pipetted from the finger tip in a quantity of 0.2 ml, which was brought into the water quantity of 0.4 ml in the apparatus within one half minute.

COAGULATION TIME TESTS

In Vivo Tests. — The normal times of coagulation were determined by a double test (methodic error: $S = \sqrt{\frac{\Sigma \Delta^2}{2n}} = 21$ seconds). Subsequently, 5 ml of camphorated oil (20% camphor in olive oil) were administered intramuscularly. The time of coagulation was then determined a) from the native blood or b) from the citrated blood, at first with greater frequency and later once every hour, during a period of 2 to 7 hours. The citrated blood was recalcified with $\frac{1}{4}\%$ calcium chloride solution.

In Vitro Tests. — After the normal tests, oil camphor was drawn into the blood sampling pipette before sampling, and successive coagulation tests were made, every time cleaning the pipette between tests by water-ether-air suction so that the camphor admixed to the blood was made to decrease by and by ad zero. It was considered important to achieve also very minute camphor additions, for the reason that one and the same substance may inhibit the coagulation in high concentrations and promote it in small concentrations (6). The tests were made both with a) native blood and with b) citrated blood.

Supplementary tests were carried out *in vivo* with native blood upon aqua camphorae injection. Moreover the effect of olive oil alone was tested *in vitro* with native blood. In order to eliminate a possible effect of pain with mobilisation of adrenalin caused by the needle prick, successive determinations of the coagulation time repeated at intervals were performed without camphor injections.

DETERMINATIONS OF PROTHROMBIN INDEX, THROMBOCYTE COUNT AND TOTAL PLASMA CALCIUM.

After the main experiments some determinations of the prothrombin index, the thrombocyte count and the total calcium of the plasma were performed before and after injections of camphor.

RESULTS

In vivo tests

a) Native blood

Table 1 shows the reduction of the normal times of coagulation upon injection of camphor oil. All normal values ranged fairly evenly between 6—7½ minutes. The shortest times of coagulation upon injection were of the order of magnitude of 1—5½ minutes, the reduction of the time of coagulation thus ranging between 83—26%. As early as 15 minutes after the injection the time of coagulation had decreased. Maximum effect was observed already at this time, or not later than within two hours. Return to the normal value occurred with two subjects only, after 5 and 2 hours, respectively (the effect even otherwise being least in the case of the latter subject). In the subject No. 4 the effect persisted in a mild degree even after 7 hours.

TABLE 1
REDUCTION UPON CAMPHOR INJECTION OF THE NORMAL TIMES OF COAGULATION
DETERMINED FROM NATIVE BLOOD

Subject No.	1 H.S.	2 S.R.	3 E.J.	4 L.A.	5 A.S.	6* Y.A.
Time of coagulation prior to camphor injection	7'00"	7'30"	7'30"	6'00"	7'30"	6'30"
Camphor injection	→	→	→	→	→	→
Times of coagulation after camphor injection						
10 min.					5'00"	
15 "		4'00"	5'30"			
20 "	2'00"				6'00"	
30—40 "	2'00"	5'00"	7'00"	5'00"		2'30"
45—60 "	4'00"	6'00"	8'30"	2'30"	5'00"	
1—1 1/2 h.	6'30"		5'30"	3'00"		3'30"
1 1/2—2 "	7'30"	6'30"	8'00"	1'00"	5'00"	
2—2 1/2 "	4'30"		7'30"	3'30"		
2 1/2—3 "	5'30"	4'30"				
3—3 1/2 "					4'00"	
3 1/2—4 "		5'00"				
4—4 1/2 "		6'00"		1'00"		
4 1/2—5 "		7'00"		3'00"		
5—5 1/2 "		7'00"				
5 1/2—6 "		7'00"				
6 1/2—7 "				4'00"		
7—7 1/2 "				5'00"		
Maximum reduction of coagulation time, %	71%	46%	26%	83%	33%	61%

* Subject No. 6 was injected with *aqua camph.* instead of *ol. camph.*

The plots obtained from the results (Fig. 1), instead of showing a uniform course with time, consisting of one dip and a subsequent rise, indicate that the reduction of the time of coagulation would occur in two phases. The curves go down twice to their minimum (or close to the same), reverting to normal values, or close to them, between the minima. In subject No. 6 5 ml of *aqua camph.* intramuscularly was given instead of *ol. camph.* A distinct reduction of the time of coagulation was observed. This appears to *justify the exclusion of the olive oil effect as a factor reducing the time of coagulation.*



Fig. 1. — Diagram showing the change of the time of coagulation with subject L.A. after injection of camphorated oil (at C).

With subject S.R. (No. 2) determinations of the time of coagulation were carried out at intervals of 15 minutes to 1 hour *without* camphor injection, the results being:

6'30", 6'30", 6'30", 7'00", 6'30", 6'30".

The times of coagulation were found to be highly uniform, whereas after injection of camphor a diphasic reduction amounting to 46% took place as shown in table 1. Therefore *the adrenalin effect caused by the pain and fright of being pricked can be left out of account.*

b) Citrated blood

It can be seen from Table 2 that the times of coagulation determined from citrated blood are of the same order of magnitude before and after the camphor injection. In this case, thus, *the camphor injection does not reduce the times of coagulation, even previously short, of the citrated blood.*

TABLE 2

TIMES OF COAGULATION DETERMINED FROM CITRATED BLOOD, RETAINING THEIR LENGTH UPON CAMPHOR INJECTION

Subject No.	1 A.S.	2 Y.A.	3 H.S.	4 Ha.S.
Time of coagulation prior to camphor injection	5'00"	4'00"	4'30"	4'00"
Camphor injection	→	→	→	→
Times of coagulation after camphor injection				
25 min.				3'30"
35—40 "	4'30"			
1—1 1/2 h.		3'30"	4'00"	4'00"
2 "				4'30"

In vitro tests

a) Native blood

TABLE 3

CHANGE OF THE TIMES OF COAGULATION UPON TREATING NATIVE BLOOD IN VITRO WITH CAMPHOR IN DECREASING CONCENTRATION

Subject	A.S.	S.R.
Nomal time of coagulation	5'30"	6'00"
Times of coagulation after admixing <i>ol. camph.</i> to native blood.		
Tests in succession according to falling concentrations.		
Oil drops on the surface	4'30"	6'00"
Cleaning pipette by suction	5'30"	
D:o	5'30"	
Suction and ether ..	5'00"	4'00"
D:o	2'30"	
D:o	3'30"	
D:o		6'00"

TABLE 4

TIMES OF COAGULATION OF NATIVE BLOOD REMAINING UNCHANGED ALTHOUGH OLIVE OIL IS ADMIXED TO THE BLOOD IN VITRO (CONTROL TEST)

Subject	A.S.	S.R.
	5'00"	6'00"
Times of coagulation after admixing <i>ol. oliv.</i> to native blood in falling concentrations.		
Oil drops	5'30"	
Cleaning pipette by suction	5'00"	
Suction and ether ..	6'00"	5'30"
D:o	5'30"	

Table 3 reveals how the *normal values* obtained with a clean blood sampling pipette *decrease when, subsequently, a suitable camphor concentration has been achieved in the pipette*. High camphor concentrations did not change the time of coagulation and reduced values were not obtained until at very low camphor concentrations. (The ether, in itself affecting the coagulation of blood, has always been well eliminated by means of air suction.)

Control tests were carried out similarly as before, except that olive oil was used instead of ol. camphor., Table 4. *No changes of the time of coagulation were observed.*

b) Citrated blood

TABLE 5

CHANGE OF THE TIMES OF COAGULATION OF CITRATED BLOOD AFTER IN VITRO
CAMPHOR TREATMENT

Subject	H.S.	S.R.	E.J.
Normal time of coagulation	4'30"	4'30"	4'30"
Times of coagulation after admixing <i>ol. camphor</i> to the <i>citrated</i> blood in decreasing concentrations			
Oil drops on the surface of the blood	4'30"	3'00"	4'00"
Suction	4'00"	0'20"	3'30"
Suction and ether	3'00"	3'00"	1'30"
D:o	4'00"	3'30"	3'00"
D:o	4'00"	4'00"	4'00"
D:o	4'00"		

Table 5 reveals that, again, in the tests with citrated blood the normal times of coagulation are lower than with native blood. However, *lower times of coagulation* are obtained for the samples drawn with a pipette treated with *ol. camphor*.

PROTHROMBIN INDEX, THROMBOCYTES AND TOTAL CALCIUM OF THE PLASMA

Prior to commencing the actual camphor tests, the prothrombin index (P.I.) and the thrombocyte count (T) of the test subjects *under normal conditions* were studied.

The prothrombin index was thus found to range between 104 and 130, the values obtained for one and the same subject differing by 2 to 20. The thrombocyte count of one and the same subject in the normal tests showed a variation by 52,000 at its most (equalling 12% of the maximum figure). The daily variations of blood calcium after meals, at rest and under stimulation are known from literature (2).

In vivo tests

TABLE 6

PROTHROMBIN INDEX, THROMBOCYTE COUNT AND TOTAL CALCIUM OF THE PLASMA BEFORE AND AFTER CAMPHOR INJECTION

Subject No.	1 A.S.			2 C.A.			3 G.W.		
	P.I.	T	Ca, mg %	P.I.	T	Ca, mg %	P.I.	T	Ca, mg %
Prior to camphor injection	104	321,000	12.2	120	251,000	12.8	110	187,000	13.2
Camphor injection →	→	→	→	→	→	→	→	→	→
After camphor injection									
15—20 min.	108	267,000	—	110	220,000	—	121	225,000	—
½ h.	110	264,000	12.2	—	—	—	121	268,000	—
1 "	108	253,000	11.0	106	200,000	11.6	118	177,000	—
2 "	124	281,000	—	104	185,000	12.0	124	178,000	—
3 "	113	262,000	11.0	110	175,000	10.6	113	190,000	12.4
4 "	124	315,000	11.2	126	170,000	11.4			
6 "				121	—	—			
20 "				118	168,000	—			
24 "				—	165,000	—			
Max. deviation from normal ..	+20	—68,000	—1.2	+6	—86,000	—2.2	+14	—10,000	—0.8
Differences, % ..		21 %			34 %				

It can be seen from Table 6 that, judging on the basis of what was said above, the deviations from normal of the prothrombin index are not significant although in the test series an increase of the values can be observed in the latter part of the series. The thrombocyte series show a distinct decrease of the count with subjects No. 1 and 2. On the whole, too, all thrombocyte counts in these two

series were below normal. With subject No. 3 no clear decrease of the thrombocyte count could be noted, on the contrary one of the values was above normal.

Even considering the great possibilities of error in the determination of the thrombocyte count and the small number of test subjects *it seems that a decrease in the thrombocyte count might occur after camphor injection, which can probably be considered an indication of thrombocyte breakdown.*

The plasma calcium values after camphor injection have been lower as a rule. However, the deviations are within the limits of error.

In vitro tests

TABLE 7

THE PROTHROMBIN INDEX OF BLOOD TREATED IN VITRO WITH OL. CAMPHOR

Subject	A.S.	C.A.	G.W.
Normal Value of P.I.	130	121	130
P.I. values after treating the blood samples in vitro with ol. camphor. in decreasing concentrations	130 124 124 124 130 130	118 124 118 121 118	100 108 108 108 104
Maximum deviation from normal	-6	+3	-30

Table 7 reveals that *no change in the prothrombin index is achieved when citrated blood samples are treated in vitro with camphor.*

Summarising the results, it can be said that in the tests with native blood a remarkable reduction of the time of coagulation, usually occurring in two phases, was obtained after camphor injection. In the tests *in vitro*, the reduction could be demonstrated in native as well as in citrated blood. No positive change in the prothrombin index occurred after camphor injection. It seems that a decrease might occur in the thrombocyte count.

DISCUSSION

The circumstance that no changes could be produced in the tests *in vivo* with *citrated blood* is probably due to the fact, in part at least, that sodium citrate in itself already promotes coagulation by breaking down thrombocytes (9). The values obtained in the normal tests were therefore reduced and no great further changes could be expected. The conformity of the tests *in vivo* and *in vitro* is thought to indicate that *the effect of camphor is one concerning the mechanism of blood coagulation* and is thus not, for instance, due to absorption of tissue fluid into the circulation. The effect of camphor on the coagulation of blood is, however, thought to be *indirect in so far that through the influence of camphor thrombocytes would be broken down which again would increase the quantity of substances promoting the formation of thromboplastin*.

The effect of enhanced coagulation is probably *linked with a certain camphor concentration in the blood*. If this optimum concentration is exceeded, no effect is obtained, but it manifests itself again when the concentration goes down. This assumption would readily explain the diphasic shortening of the coagulation time after a large dose of camphor. Smaller camphor doses than those employed in this work would thus be more advisable in practice.

As has been mentioned before, camphor has been used as a haemostyptic in tuberculous pulmonary haemorrhages. In such cases, as a rule, the cause of the haemorrhage is a wall lesion. However, according to Künzer (5), also the function of the thrombocytes and the quality of the thromboplastin seem to be concerned. The material in the present work consisted of healthy subjects only. It would be interesting to note the kind of effect camphor would have in a material similar to that of Künzer.

The investigations treated in this paper speak in favour of the contention that the *use of camphorated oil at the occurrence of pulmonary haemorrhage is fully motivated on account of its effect of enhancing the coagulation of blood*. On the other hand it appears that camphor is one of the substances that should be avoided when there is a risk of thrombosis.

SUMMARY

The mechanism of the haemostypnic effect of camphorated oil has been investigated. Intramuscularly administered, it has been found to reduce the time of coagulation in a remarkable degree, the reduction occurring in two phases. Also in tests *in vitro* such a reduction could be produced if the camphor concentration was sufficiently low. Experiment with aqua camphor. *in vivo* also produced the same result. Upon camphor injection no positive change occurred in the prothrombin index, whereas a reduction of the thrombocyte count may occur. No effect upon blood calcium could be noted. — The reduction of the time of coagulation is thought to be caused by a possible break-down of thrombocytes, resulting in a higher content of thromboplastin-producing substances in the blood.

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AUS DEM WIHURI-FORSCHUNGSIINSTITUT UND AUS DER WISSENSCHAFTLICHEN
ABTEILUNG DER ARZNEIMITTELFABRIK »LÄÄKETEHdas ORION OY»
HELSINKI, FINNLAND

ÜBER DIE WIRKUNG VON PALFIUM (R 875) AUF DAS ADRENALIN-LUNGENÖDEM DER MAUS

von

VILJO ANTILA und GOTTFRIED HÄRTEL

(Bei der Schriftleitung eingegangen am 4. Dezember, 1958)

Morphium hat bekanntlich eine hemmende Wirkung auf das experimentelle Lungenödem. Luisada (3, 4) hat festgestellt, dass Morphium das mit Adrenalin hervorgerufene Lungenödem bei Kaninchen zu verhindern vermag und Poulsen (5) hat nachgewiesen, dass es bei Mäusen gegen das durch Kohlendioxyd hervorgerufene Lungenödem wirksam ist.

Seit einiger Zeit wird zur Bekämpfung starker Schmerzen ein neuer von Janssen und Mitarbeitern (1) synthetisierter Stoff angewandt. Es handelt sich hierbei um N(1,1-diphenyl-2-methyl-3-morpholinbutyryl)-pyrrolidin.bitartrat (R 875), das in verschiedenen Ländern unter dem Namen Palfium¹ im Handel ist. Folgende Untersuchungen wurden durchgeführt zur Klärung der Frage, ob Palfium einen Einfluss auf das experimentelle, mit Adrenalin verursachte Lungenödem besitzt.

METHODEN

Für die Versuche wurden vierzig männliche weisse Mäuse verwandt, deren Gewicht 17,0 g bis 22,5 g betrug. Die Tiere wurden in vier Gruppen zu zehn Tieren eingeteilt. Gruppe 1 waren die Kontrollen und wurden durch intraperitoneale Injektion von 0,2 ccm einer 25%igen Kaliumcyanid-

¹ Palfium, R 875 wird in Finnland von der Arzneimittelfabrik Lääketehdas Orion Oy hergestellt.

lösung getötet. Sämtliche andere Tiere (Gruppe 2, 3 und 4) erhielten intraperitoneal 5 mg/kg Körpergewicht Adrenalinhydrochlorid und wurden 15 Minuten später in gleicher Weise getötet wie die Kontrolltiere. Um die Wirkung von Morphinum und Palfium zu prüfen, erhielten die Tiere der Gruppe 3 20 Minuten vor der Adrenalininjektion subcutan Morphinumhydrochlorid 6 mg/kg Körpergewicht, die Tiere der Gruppe 4 Palfium 1,5 mg/kg Körpergewicht.

Sofort nach dem Töten wurden die Lungen in der von Poulsen (6) angegebenen Weise herauspräpariert, und nach vorsichtigem Abtupfen von Blut innerhalb von 2 bis 3 Minuten gewogen. Das Lungenödem wurde als Gewicht der Lungen in mg/g Körpergewicht bestimmt. Die Ergebnisse wurden statistisch ausgewertet.

ERGEBNISSE

Während des Versuches starb keins der Tiere. Als Zeichen des eingetretenen Lungenödems trat besonders bei den Tieren der Gruppe 2, die nur Adrenalin erhalten hatten, blutiger Schaum aus Nase und Mund hervor. Das Straub-Hermannsche Mäuseschwanzphänomen war bei den Palfiumtieren (Gruppe 4) erheblich stärker ausgeprägt als bei den Tieren, die Morphinum erhalten hatten (Gruppe 3). Auch erschienen die Mäuse nach der Palfuminjektion viel aufgeregter als nach der Morphinumgabe.

TABELLE 1.

Gruppe	Anzahl der Tiere	Mittl. Ge- wicht in g ± mittl. Fehler	Mittl. Lun- gengewicht in mg ± mittl. Fehler	Mittl. Lun- gen- gewicht in mg/g Körpergewicht ± mittl. Fehler
I Kontrollen	10	21,1 ± 0,6	144,3 ± 4,1	6,91 ± 0,10
II Adrenalin 5 mg/kg intraperitoneal	10	19,0 ± 1,7	244,4 ± 22,9	12,82 ± 1,06
III Morphium 6 mg/kg s.c. Adrenalin 5 mg/kg i.p.	10	18,5 ± 0,4	177,7 ± 10,2	9,69 ± 0,66
IV Palfium 1,5 mg/kg s.c. Adrenalin 5 mg/kg i.p.	10	18,4 ± 0,4	156,8 ± 6,4	8,64 ± 0,47

Die mittleren Tiergewichte und die mittleren absoluten und relativen Lungengewichte der vier Gruppen sind in der Tabelle 1 enthalten.

Adrenalingabe (Gruppe 2) führte innerhalb von 15 Minuten fast zu einer Verdoppelung des mittleren relativen Lungengewichtes. Diese Gewichtszunahme ist hoch signifikant ($p < 0,001$).

Bei vorhergehender Morphiumgabe (Gruppe 3) nahm das relative Lungengewicht nach der Adrenalininjektion weniger zu. Der Unterschied zwischen den Werten der Gruppe 2 und 3 ist wahrscheinlich signifikant ($p = 0,023$). Palfium (Gruppe 4) wirkte im vorliegenden Versuch der Gewichtszunahme durch Adrenalin noch stärker entgegen, so dass ein signikanter Unterschied zwischen der Gruppen 2 und 4 vorliegt ($p < 0,01$). Zwischen der Morphium- und der Palfiumgruppe war kein signikanter Unterschied feststellbar ($p = 0,2$).

Weder Morphium noch Palfium vermochte das durch Adrenalin verursachte Lungenödem völlig zu verhindern: Gegenüber den Kontrollen waren die mittleren relativen Lungengewichte beider Gruppen deutlich erhöht ($p < 0,01$).

BESPRECHUNG

Wie Morphium hat auch Palfium eine hindernde Wirkung auf das mit Adrenalin verursachte Lungenödem der Maus. Diese Wirkung Palfiums scheint stärker ausgeprägt zu sein als die von Morphium, da beide Stoffe im Verhältnis 1 : 4 angewandt ungefähr den gleichen Effekt zeigen. Dies stimmt mit den Ergebnissen von Tierversuchen überein, in denen Palfium eine stärkere analgetische Wirkung als Morphium gezeigt hat und zwar bei Ratten eine 5,8 mal, bei Mäusen eine 12 mal stärkere Wirkung (2). Die Morphiumdosis 6 mg/kg Körpergewicht wurde auf Grund der Arbeit von Poulsen (5) gewählt. Der Ausgangspunkt für die Wahl der Palfiummenge war, dass die beiden Stoffe klinisch im Verhältnis 1 : 2 — 1 : 4 angewandt werden.

Die Adrenalindosis 5 mg/kg Körpergewicht betrug nur die Hälfte von der, welche Poulsen in seiner Arbeit angewandt hat. Sie wurde auf Grund eines Vorversuches festgelegt, der ergeben hatte, dass 10 mg/kg Körpergewicht bereits so toxisch war, dass ein grosser Teil der Tiere dadurch starb.

Das Töten der Tiere mit einer intraperitonealen Injektion von Kaliumcyanid hat sich für die Untersuchung von experimentellem Lungenödem als besonders geeignet erwiesen [Poulsen (6)].

Ob die hier aufgezeigte hindernde Wirkung von Palfium auf das experimentelle Lungenödem Bedeutung für die Therapie des Lungenödems besitzt, kann nur durch klinische Untersuchungen entschieden werden.

ZUSAMMENFASSUNG

Die Wirkung des neuen Analgeticum Palfium = R 875 N (1,1-diphenyl-2-methyl-3-morpholinbutyryl)-pyrrolidin.bitartrat auf das mit Adrenalin verursachte Lungenödem der Maus wurde untersucht und mit der entsprechenden Wirkung von Morphin verglichen. Palfium (1,5 mg/kg Körpergewicht) zeigt den gleichen hindernden Einfluss wie Morphin (6 mg/kg Körpergewicht). Zur Bestimmung der Schwere des Lungenödems wurde das relative Gewicht der Lungen benutzt. Die Ergebnisse wurden statistisch gesichert.

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HAEMAGGLUTINATION CAUSED BY PNEUMOCOCCI

by

VEIJO RAUNIO

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INTRODUCTION

In common with viruses, many bacteria have the ability to agglutinate erythrocytes. Examples of such bacteria are certain *Staphylococcal* strains, *Escherichia coli*, *Haemophilus pertussis*, *H. parapertussis*, *H. bronchisepticus*, *Vibrio comma* and *Shigella alkalescens*.

Also pneumococci are able to agglutinate red cells. The first to study this effect was Hallauer who reported in a paper published in 1946 that pneumococcus suspensions, particularly suspensions of organs of mice which had suffered from pneumococcal septicaemia, gave a positive Hirst test (1). The haemagglutinating factor was found to be thermolabile and the pneumococcus antisera (anti- I and anti- II) prevented the haemagglutination caused by all the various pneumococcus types. Löffler (2) continued these investigations and showed that the haemagglutinin is probably identical with the autolytic enzyme isolated from pneumococcus which had been previously described by Dubos (3). According to Löffler chick erythrocytes were more readily agglutinated than those of rodents and various mammals, although the red cells of some chickens were not agglutinated at all.

Attention was called to this phenomenon in connection with virus investigations when it was noted that a pneumococcal strain that had infected the allantois of a chick embryo was able to agglutinate chick red cells. Since this phenomenon has been relatively little studied, a more close investigation of the phenomenon has been carried out.

MATERIAL AND METHODS

Strains. — The pneumococcus strains employed had been isolated from sputum and pus samples that had been sent to the diagnostic laboratory. The strains were typed and kept virulent by passage in mice. The haemagglutinating powers of 17 types most commonly encountered in this country (types I, III—XI, XV—XIX, XXIII, XXVII) were examined.

The R variant was prepared from type I with the aid of anti-serum according to the directions of Paul (4). The streptococcus type strains had been obtained from the National Collection of Type Cultures.

Sera. — The 100 human sera used in the antibody determinations were chosen at random from those sent to the diagnostic laboratory.

Culture of Pneumococci. — Since preliminary experiments showed that pneumococci that had been cultured in chick embryos produced a very active haemagglutinating factor and that the virulence of the pneumococci did not weaken during egg passage, chick embryos were mostly employed in the culture of the pneumococcus strains.

A pneumococcal culture grown in blood agar was suspended in physiological saline and 0.2 ml of the suspension was injected into the amnion or allantois of chick embryos 7—12 days old. The growth of pneumococci inoculated directly from the colonies with a platinum loop did not progress as well as when suspensions were injected. The egg passages were continued by injecting 0.2 ml of the allantoic fluid into the allantois of new embryos. The injected eggs were incubated 1—4 days at 37°C and cooled to +4°. A sample of the allantoic fluid was taken from each egg and stored separately. The growth of the pneumococci was checked by transfer to blood agar plates.

Haemagglutination Tests. — The haemagglutination tests were carried out on plastic plates as described earlier by Salk (5). A suspension of 0.5 per cent chicken cells in saline was used. For the titration 0.25 ml volumes of the allantois fluids infected with pneumococci were diluted in twofold steps with equal volumes of saline and 0.25-ml of the 0.5 per cent chicken cell suspension was added to each dilution. The samples were placed in depressions (of the plate) and shaken thoroughly and the results read after the

agglutinated cells had settled to the bottoms of the depressions at room temperature. The end point was taken to be the dilution giving maximum agglutination, which was considered to have resulted when a uniform film of cell clumps covered the entire bottom of the depression. In the negative depressions there was a central, sharply outlined disc of cells. Intermediate degrees were scored \pm . In the haemagglutination inhibition tests 4 agglutinating units of antigen were used. The antiserum dilutions were prepared with a 0.5 per cent cell suspension in twofold series from 1: 4 to 1: 2048. The results of the tests were evaluated by examining the cell deposits at the bottoms of the depressions. The recorded titre was the reciprocal of the last dilution of serum that gave complete inhibition of agglutination.

The autolytic pneumococcal enzyme preparation used in the study was prepared and its activity checked as described by Dubos (6).

RESULTS

Growth of Pneumococci in the Embryos. — Regardless of type the pneumococci grew best in the allantoic sacs of embryos 7—8 days old. When the strain was very virulent (a culture diluted 1: 10⁸ killed a white mouse within 24 hours), very little difference in growth was noted in embryos from 8 to 11 days old. No difference in virulence was observed when the pneumococci were introduced into the amniotic sac instead of the allantois. Generally the 7—8-day-old embryos died within 24 hours, but a part of those from 9 to 12 days old survived longer (2—6 days). As criteria of growth, the turbidity of the allantoic fluid and the number of colonies on a blood agar plate were employed.

Haemagglutination. — The haemagglutination titres of the allantoic and amniotic fluids varied between 1: 32 and 1: 2048. The titre depended on the growth of the pneumococci and was most uniformly high in those embryos which were inoculated with the pneumococcus strain on the seventh or eighth day of incubation. The avirulent strains and the R variant grew poorly in living embryos (the growth was slow and the embryo survived) and gave haemagglutination titres that were low or could not be determined, but they fared well in the amniotic sacs of embryos killed by refrigeration and produced abundant haemagglutinin. As the

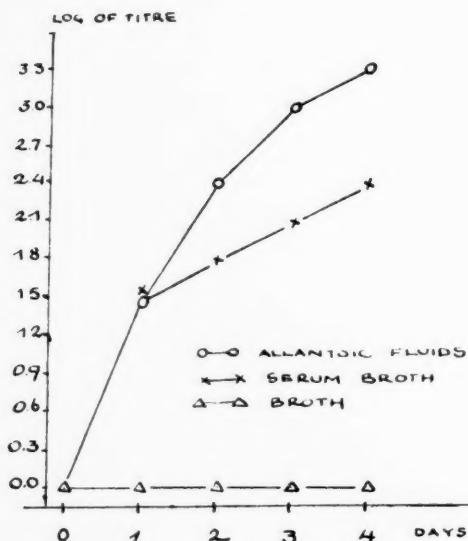


Fig. 1. — The production of haemagglutinin of pneumococci cultured in various media

TABLE 1

THE RESULTS OF HAEMAGGLUTINATION TESTS CARRIED OUT WITH HUMAN AND ANIMAL CELLS AT VARIOUS TEMPERATURES

Red Cells	+4°		+20°		+37°		+56°	
	Pneumoc. Culture	Saline	Pneumoc. Culture	Saline	Pneumoc. Culture	Saline	Pneumoc. Culture	Saline
Human A	—	—	—	—	—	—	—	—
B	—	—	—	—	—	—	—	—
AB	—	—	—	—	—	—	—	—
O	—	—	—	—	—	—	—	—
Pig	—	—	—	—	—	—	—	—
Sheep	—	—	—	—	—	—	—	—
Cow	—	—	—	—	—	—	—	—
Rabbit ..	—	—	—	—	—	—	—	—
Guinea Pig	—	—	—	—	—	—	—	—
Hen	+1: 16	—	+1: 32	—	+1: 64	—	+1: 128*	—

* The haemagglutination occurred before the inactivation of the agglutinating factor.

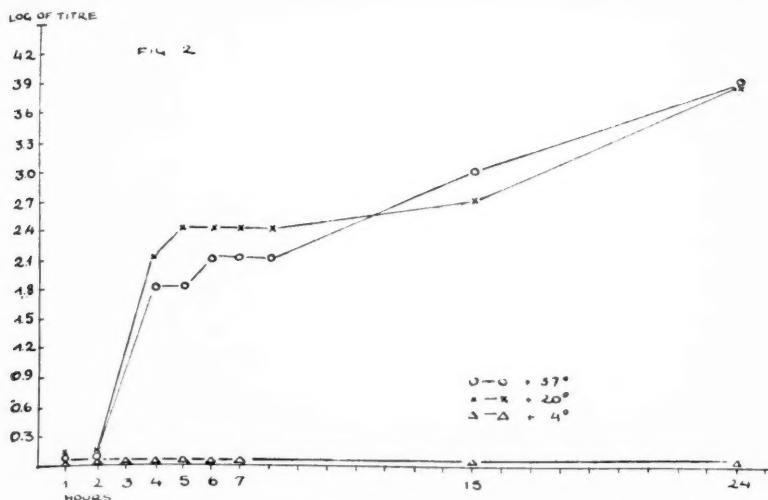


Fig. 2.—Effect on the titre of mixing of the haemagglutinin dilution series.

period of pneumococcal infection became longer, the haemagglutinin titre increased (Fig. 1). The haemagglutinating power of a pneumococcus strain that had been transferred thirteen times remained unchanged. The strains were subcultured at intervals of one week and the haemagglutinin titres were determined after each passage.

When the agglutination was performed at different temperatures ($+4^\circ$, $+20^\circ$, $+37^\circ$, $+56^\circ$), it was observed that it took place more rapidly and the titre tended to rise with increasing temperature (Table 1). When cells of the same dilution series were again shaken with saline, the titre increased after each mixing at room temperature ($+20^\circ\text{C}$) and at $+37^\circ$, but, as Löffler had already noted, remained unchanged at $+4^\circ\text{C}$ (Fig. 2). The haemagglutinating factor was inactivated at $+56^\circ$. The titre of a pneumococcus-infected allantoic fluid stored in a refrigerator ($+4^\circ$) has remained unaltered over two years. (It may be mentioned that it was possible to isolate the live pneumococcus strain from most of the pneumococcus-infected allantoic fluids that had been stored one month at $+4^\circ$). When the pneumococci were centrifuged from the allantoic fluid, the agglutinating factor was retained in the fluid. This fluid was able to lyse gram-positive pneumococci that had been killed by heating.

TABLE 2

THE RESULTS OF HAEMAGGLUTINATION TESTS CARRIED OUT WITH RED CELLS FROM VARIOUS HENS.

Hen	Titre of Haemagglutination	Saline and Cells
1	—	—
2	1: 32	—
3	1: 256	—
4	—	—
5	1: 512	—
6	1: 32	—
7	—	—
8	1: 128	—

An enzyme preparation prepared from pneumococci as described by Dubos and which autolysed the pneumococci to a gram-negative detritus and agglutinated chick red cells at a titre of 1: 16. The preparation was inactivated by heating 30 min. to +56°C.

Haemagglutination tests in which human, bovine, pig, sheep, rabbit, guinea pig and chick red cells were employed and which were carried out at +4°, +20°, +37° and +56° revealed that only red cells of 5 of 8 chicks were agglutinated (Tables 1 and 2). Attempts to elute the haemagglutinating factor from the agglutinated chick red cells were unsuccessful.

Pneumococci produced the haemagglutinating factor also when cultured in serum broth and liver broth, but the titres were much lower (1: 4—1: 64) than when the pneumococci grew in the allantoic fluid.

Inhibition of Haemagglutination. — It is well known that a homologous antiserum inhibits specifically the haemagglutination caused by an influenza virus. The pneumococcal antisera inhibited haemagglutination caused by pneumococci with titres 1: 64—1: 128, but the same inhibition was effected also by normal rabbit serum as well as by *E. coli* and *Salmonella* antisera. The inhibition is hence neither type- nor even strain-specific. Inhibition titrations carried out with the 100 randomly selected human sera did not yield divergent results, for all inhibited haemagglutination with titres 1: 64—1: 256.

Haemagglutination Tests with Streptococci. — In view of the close relationship between streptococci and pneumococci it was

of interest to perform haemagglutination tests also with the former. The following strains were employed in the tests: Str. types A, B, C, D, E, F, G, H, L, M, Str. *uberis*, Str. *agalact.*, Str. *dysgalact.*, Str. *sanguis*, Str. *cremoris*, Str. *salivarius*, Str. MG, Str. *bovis* and ten unidentified α -haemolytic streptococcus strains. None of these agglutinated chick red cells.

DISCUSSION

The haemagglutination caused by pneumococci differs essentially from that effected by viruses. It was not found possible to elute the pneumococcal haemagglutinins from the red cells and the rise in titre noted when the mixtures of red cells and pneumococcal suspensions were shaken at intervals suggests that more agglutinins were released into the solution from the pneumococci. In the haemagglutination brought about by viruses the opposite is true: agglutinins are eluted from the agglutinated cells which become inagglutinable and the titre decreases after each shaking. When Löffler shook a suspension of pneumococci to which no red cells had been added, he observed that the titre rose after each shaking. Pneumococci lost their agglutinating power when killed by rapid heating to $+75^\circ$. These observations suggest that the agglutination is closely connected with the autolysis of the pneumococci. Since, furthermore, an autolysate prepared from pneumococci was found to agglutinate red cells and lyse pneumococci and lost this property when warmed for ten minutes at $+56^\circ$, it may be concluded that the autolytic enzyme of pneumococci and the haemagglutinating factor are identical.

None of the investigated streptococcus strains was able to agglutinate chick red cells. Since, on the other hand, all of the 25 pneumococcal strains examined agglutinated chick red cells, it may be possible to use this method to distinguish between pneumococci and α -haemolytic streptococci with similar morphological features.

During the course of the experiments it became apparent that chick embryos are very suitable media for the culture of pneumococci. Knothe (7), who studied the growth of aerobic bacteria in chick embryos, also came to the same conclusion with respect to pneumococci.

SUMMARY

Cultures of pneumococci grown in chick embryos from 7 to 12 days old, and especially in those 7—8 days old, were found to agglutinate chick red cells. The haemagglutinin titres varied for red cells from different chickens, and cells of some chickens were not agglutinated. The quantity of haemagglutinins produced increased with the time the embryo was infected with pneumococci. The agglutination became more rapid and the titre rose as the temperature was increased. When suspensions of red cells in serial dilutions of allantoic fluid from infected embryos were repeatedly shaken the titre increased continuously with increasing dilution. The pneumococcal haemagglutinin is thermolabile and probably identical with the pneumococcal autolytic enzyme. Pneumococcal antisera do not possess this specific power of inhibiting haemagglutination.

None of examined α - and β -haemolytic streptococcus strains haemagglutinated chick red cells.

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FROM THE STATE MEDICAL BOARD,
DEPARTMENT OF HYGIENE AND EPIDEMIOLOGY

DIPHYLLOBOTRIASIS AS AN INFECTIOUS DISEASE IN FINLAND

by

T. LAINE, RISTO PÄTIÄLÄ, M. E. PARMALA, O. LOKKI

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Diphyllobothriasis occupies a special position among the parasitic infestations of the human intestine in Finland. Other infestations such as ascariasis, oxyuriasis and especially taeniasis occur on a small scale. National habits in fish consuming must be regarded as the most important reason for the high incidence of diphyllobothriasis. Many fresh water species, *e.g.* pike, perch, burbot, salmon, whitefish, trout and *Coregonus albula* (a small species of white fish), are intermediate hosts for *diphyllobothriocephalus*. These fish are common in Finland's water systems. This fact combined with the great number of sweet water waterways in the country helps to spread the infection to sections of population which eat fresh, inadequately prepared — raw or undersalting — fish.

Several investigations have been conducted in Finland on the incidence of diphyllobothriasis, based on questionnaires sent to physicians or on studies of certain sections of the population for the presence of tapeworm ova in the faeces.

INCIDENCE ON THE BASIS OF QUESTIONNAIRES SENT TO DOCTORS

The oldest of the materials is that collected by Sievers (8) on the basis of a questionnaire circulated to the medical profession of the country. The picture it provides, however, is summary,

especially as the number of physicians was then very limited. Ehrström's statistics of 1926 (1) give a more detailed picture of the situation. The majority of persons infected live, according to him, in Eastern Finland, in the coastal communes in the west and in Northern Finland. The smaller incidence of infestation in the statistics for the northernmost parts of the country may be ascribed to the shortage of public health personnel in those regions. The most recent statistical material based on questionnaires is that collated by Huhtala (3). It, too, shows Eastern Finland, especially the eastern parts of the administrative district of Mikkeli, to be the most severely infested. According to this material, the localities of the western coast of Ostrobothnia are relatively unaffected and there is only a minor concentration in the Kokemäenjoki valley in Northern Satakunta. The infestation of Northern Finland is smaller in Huhtala's than in Ehrström's statistics. According to Huhtala, tapeworm infestation increases towards the end of the summer, and this he attributes to the fact that the larvae (*coracidium*) can only develop during the three summer months.

INVESTIGATION OF CERTAIN SECTIONS OF THE POPULATION

Several statistics have been compiled from studies based on laboratory tests and concerned with certain, limited sections of the population (Table 2). The majority have dealt with conscripts or war-time soldiers. In all of the investigations of this type (2, 5, 6, 10 and 12) the material has consisted principally of persons of c. 20 or 20—45. A part of Tötterman's (10) material took in civilian patients in Eastern Finland. Mustakallio (4) had the largest material but it was composed of several fairly small sections which made the whole unhomogeneous, and this must be taken into consideration in the evaluation of the results. Setälä's (7), Tähti's (9) and Uurasmaa's (11) materials covered the population of a given locality. The others included all age classes, the one of Tähti (9) only school children of 7—15. Judging by the materials consisting of conscripts and reservists throughout the country, the infestation percentage is 14—21.7 per cent; according to Mustakallio's (4) material representing the civilian population of the entire country it is 23.7; in Tötterman's (10) civilian material from Eastern Finland the figure is 41.4—66 per cent.

TABLE 1

INCIDENCE OF TAPEWORM ON THE BASIS OF PERSONAL QUESTIONNAIRES TO PHYSICIANS

Sievers	1905	very common in the greatest part of the country
Ehrström	1926	an average of 20—25 per cent of the total population, Eastern Finland 50 per cent
Huhtala	1950	an average of 20.15 per cent, Eastern Finland 61—100 per cent

TABLE 2

INCIDENCE OF TAPEWORM ACCORDING TO STUDIES OF CERTAIN SECTIONS OF THE POPULATION

			Total	
Seppä	1927	patient material of a military hospital	3,937	11.3 %
Mustakallio	1940	resumé of different statistics	15,192	23.7 »
Ollilainen	1943	a Northern Finnish regiment	2,768	31.1 »
Tötterman	1944	military hospital	850	17.8 »
		civilian patients	233	66 »
		civilian out-patient department		41.4 »
		the following year	2,006	14.5 »
Setälä	1945	Kärkölä	2,097	59 »
Gylling	1949	military records	14,631	18 »
Tähti	1952	Hankasalmi elementary school	1,226	15.8 »
Venho-Venho	1953	conscripts	957	21.7 »
Uurasmaa	1958	Inari (15 per cent of population)	988	6.2 »
		Saarijärvi (5 per cent of population)	592	6.3 »

As the above investigations give estimates based on hypotheses or are concerned only with a certain section of the population, they inevitably fail to give a reliable overall picture of the incidence of infestation, the more so as they are not based on reliable statistical sampling methods. To correct the picture given by the above investigations, we adopted an additional criterion for tapeworm incidence and studied the situation on the basis of sales of common antihelminthics sold in Finland. This gives a new view, independent of the above bases of evaluation, of the incidence of tapeworm.

As controls, in addition, statistical data have been collected in the last few years at the instigation of the Medical Board from physicians throughout the country in connection with the statistics on infectious diseases compiled monthly.

STATISTICS BASED ON THE SALES OF ANTIHELMINTHICS

The data covered the sale of various antihelminthics to dispensing chemists in 1950—52. All the drugs used to expel fish tapeworm were included. The sales quantity obtained was divided by the average number of doses to give a summary worm chart. As sales are often concentrated in the densely-populated communities of the administrative districts the figures are given not by communes but by the much larger unit of administrative districts. Table 3 gives the results.

TABLE 3

SALE OF ANTIHELMINTHICS ACCORDING TO THE DOSE NEEDED FOR EXPULSION, BY ADMINISTRATIVE DISTRICTS, 1950—52, AND THE INFESTATION PERCENTAGE CALCULATED FROM THE SALES COMPARED WITH STATISTICAL DATA BASED ON HUHTALA'S ENQUIRIES

Administrative District	Doses				Infestation % Calculated from the Use, of Antihelminthics Average	Infestation % According to Huhtala 1950
	1950	1951	1952	Total		
Uusimaa	8,247	7,013	7,980	23,240	1.1	c. 1
Turku and Pori	8,869	8,164	6,052	23,081	1.2	* 2
Aland Islands	143	118	76	337	0.5	under 1
Häme	8,292	6,566	5,992	20,850	1.2	c. 2
Kymi	10,798	12,464	9,358	32,620	3.4	20
Mikkeli	17,773	22,940	20,499	61,212	8.3	60—70
Kuopio	27,413	49,820	45,890	123,123	8.6	60—70
Vaasa	10,387	12,579	9,825	32,791	1.8	6—7
Oulu	16,307	24,192	23,921	64,120	5.9	c. 40
Lapland	7,948	7,909	7,402	23,259	4.5	* 40
Whole country	116,173	151,765	136,995	404,933	3.3	

It will be seen from the table that the statistics based on sales and the statistics based on Huhtala's questionnaires (3) agree fairly well by administrative districts. In districts where Huhtala found the incidence of infestation high the sales of antihelminthics were also higher than in administrative districts where the parasitic infestation was small in scale. However, the spread of the figures given by Huhtala is bigger than that of the sales figures. May be the people living in the infested areas are rather indocent and don't consider it necessary to expel the worms. Comparison by years

shows that sales vary in different years in the infested areas, *i.e.* the administrative districts of Kymi, Mikkeli, Kuopio and Oulu, while the vermifuge sales decrease annually in areas with a small incidence of worms. Judging by the sales, an average of 3.3 per cent of the country's population takes medicinal expulsion measures in a year. By calculating the average price of the average dosage we obtain the sums of money spent yearly on antihelminthics. The population of the slightly infested area spends an average of only c. 1.00—1.50 marks per head while in average 10 marks per head are spent in the more seriously worm-infested areas. A total of over 48 million marks has been expended in the country as a whole in three years, *i.e.* c. 4 marks per head per annum. Although antihelminthics are generally cheap, tapeworm expulsion constitutes a considerable item of expenditure. This especially if we take into consideration the fact that in addition to the price of the drugs many of the infested persons also have hospital fees or other expulsion expenses to meet as well as a loss of earnings during the period of expulsion a small part of the patients have also the expense of treatment for the complicating diseases caused by the worms.

SURVEYS BASED ON STATISTICS OF THE MEDICAL BOARD

The monthly statistics collected by the Medical Board have included since June 1954 data on the occurrence of tapeworm. The first six months may be regarded as a period during which doctors adapted themselves to this new liability. Although the statistics are not absolutely reliable even now, they give a relative idea of the incidence of worms in Finland. Variations in individual localities may be due to the changing of the communal medical officer (*cf.* Table 6), and great differences between neighbouring localities where conditions would suggest that there is actually an equal infestation percentage may be attributed to the varying degrees in interest shown by physicians in tapeworm and its expulsion. As regards some towns and rural communes, it may be of some significance also that patients report for some reason or another to certain centres or to certain doctors and thus cause an unreasonably high infestation percentage in the statistics of the locality in question. As an example of the incidence of tapeworm infestation, Fig. 1, gives the incidence per 100,000 inhabitants in



Fig. 1. — Cartogram showing the tapeworm infestation in Finland in 1957, per 100,000 inhabitants, according to the statistics of the State Medical Board.

TABLE 4

TAPEWORM INCIDENCE BY ADMINISTRATIVE DISTRICTS ACCORDING TO STATISTICS
OF THE MEDICAL BOARD, PER 100,000 PERSONS

Administrative district	1955	1956	1957	Average
Uusimaa	0.2	0.8	1.09	0.7
Turku and Pori	1.7	2.2	2.8	2.2
Aland Islands	—	0.1	0.03	0.04
Häme	0.4	1.3	1.5	1.1
Kymi	5.2	7.8	8.4	7.1
Mikkeli	10.0	14.9	18.0	14.3
Kuopio	32.5	31.2	27.1	30.3
Vaasa	1.5	3.5	4.6	3.2
Oulu	11.1	14.0	13.1	12.7
Lapland	14.2	16.4	18.6	16.4

TABLE 5

INCIDENCE OF TAPEWORM IN SOME INDIVIDUAL LOCALITIES ACCORDING TO NOTIFICATIONS TO THE MEDICAL BOARD, PER 100,000 INHABITANTS

	1955	1956	1957
Anttola	81.0	160.0	187.1
Hartola	1.0	5.0	41.4
Hyrynsalmi	50.5	53.8	59.5
Iломantsi	58.2	48.4	55.9
Joensuu	269.6	261.4	171.2
Kajaani	—	18.8	31.7
Kemijärvi	35.2	35.2	62.9
Kihtelysvaara	46.1	38.1	38.1
Kuivaniemi	27.5	36.8	27.3
Nurmes	28.3	63.8	69.8
Pelkosenniemi	30.8	36.4	33.2
Puolanka	38.0	40.5	23.0
Puumala	14.9	21.1	43.5
Pyhäselkä	52.5	57.4	27.4
Ristijärvi	34.5	43.1	49.8
Ruokolahti	15.3	39.5	28.4
Siiökainen	21.3	55.2	13.8
Suomusjärvi	—	9.3	88.3
Suomussalmi	36.3	43.9	47.5
Säynätsalo	11.6	18.8	15.8
Tervo	72.9	38.9	41.8
Tornio	40.8	30.5	25.1
Vaala	52.8	37.4	43.1

1957 calculated from monthly returns to the Medical Board. Table 4 gives the incidence per 100,000 inhabitants in the different administrative districts in each year and the average for the 3-year period. Judging by this table, infestation is highest in the administrative districts of Kymi, Mikkeli, Kuopio, Oulu and Lapland. In most administrative districts the figures increase during the observation period, probably indicating an increase in the activity of the physicians and in the readiness to report for examination. A distinct drop, on the other hand, is noted in the administrative district of Kuopio which may be proof of the fruitfulness of the propaganda campaign waged.

In Table 5 are listed the individual localities in which tapeworm infestation is greatest or in which there has been an exceptional decrease or increase in the notified cases of infestation. In the

TABLE 6

LOCALITIES IN WHICH THE COMMUNAL MEDICAL OFFICER CHANGED DURING THE PERIOD IN WHICH THE MEDICAL BOARD HAS COLLECTED STATISTICAL DATA, A CIRCUMSTANCE THAT HAS OBVIOUSLY RESULTED IN THE DIFFERENCE IN THE ANNUAL FIGURES

	1955	1956	1957
Ikaalinen	7.4	35.4	36.1
Juuka	52.4	25.9	20.4
Kolari	4.4	23.7	40.5
Konginkangas	23.3	21.9	46.9
Kuusamo	35.5	41.6	15.1
Maksamaa	—	24.1	54.9
Saari	68.7	56.7	2.4
Savonranta	5.9	42.0	69.6
Savukoski	2.1	26.7	37.2
Taivalkoski	—	28.0	38.5
Tuupovaara	21.1	32.0	43.5
Tuusniemi	21.1	16.1	39.7
Yli-Ii	11.6	27.2	38.6
Äänekoski	3.1	24.0	41.5

localities listed in Table 6 the communal medical officer has changed during the period in question and this has obviously affected the infestation figures for the different years; the new medical officer's interest in tapeworm or the readiness to report for examination has differed from that of his predecessor.

CONCLUSIONS

The diagram summarising the statistical data (Fig. 2) is based on antihelminthic sales, new cases of infestation notified to the Medical Board, the material compiled from studies (12) and Huhtala's statistics (3) compiled from enquiries. The percentual sales of antihelminthics form the ordinate. Along the abscissa are plotted the data based on the various other statistics mentioned. Scales were calculated for the different infestation statistics as follows: all the infestation values for Kuopio, (which was the most polluted district, judging by the antihelminthic sales) are put to the same point on the abscissa. The following findings emerge from the diagram:

- (a) 1.—1.2 per cent sales corresponds to a c. 0—2 per cent infestation with parasites in the population;

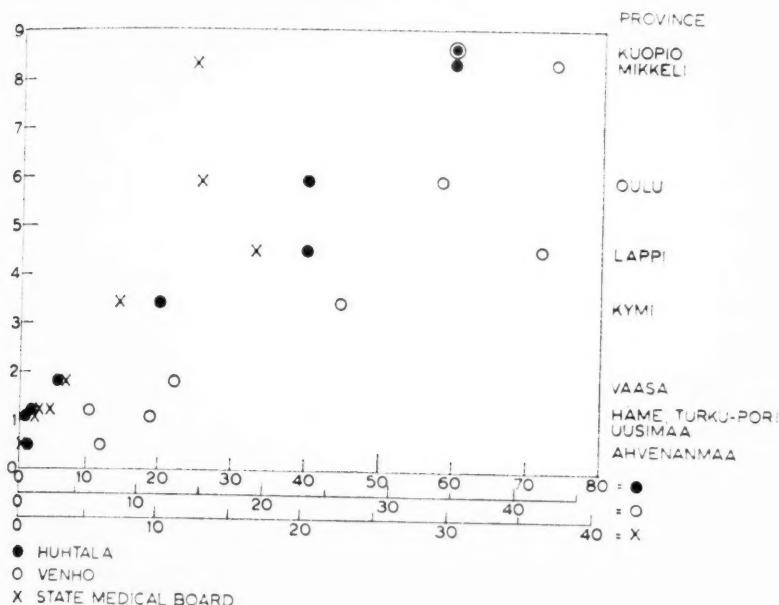


Fig. 2. — Diagram summarising the statistical data based on antihelminthic sales (cures per cent of population per annum), new cases of infestation (per 100,000 inhabitants) notified to the State Medical Board, the material compiled from laboratory studies (Venho) and Huhtala's statistics compiled from enquiries (both expressing the infestation percentage of population). The percentual sales of antihelminthics form the ordinate, infestation figures are plotted along the abscissa. For nearer explanation see text.

(b) The manifestly vigorous medicinal expulsion of worms in the district of Mikkeli in 1950—52 has, if we compare Venho's and Huhtala's estimates with the statistics of the Medical Board, decreased the incidence of worms. There is indeed a possibility that the persons infested no longer consult a physician or they frequently expel the parasites without medical assistance.

(c) The same is observed but less distinctly in the administrative district of Oulu;

(d) In the administrative district of Lapland the use of anti-helminthic cures is on too small a scale and probably calls for more effective work of enlightenment there;

Among the clearly infested areas, the situation has improved slightly in the last few years in the administrative districts of Kuopio, Kymi, Mikkeli and Oulu. More effective enlightenment work on the subject is needed in the administrative districts of Lapland.

Although the estimates of infestation frequency based on enquiries are the least reliable — notifications by doctors concern only the cases they diagnose — statistics based on direct examination are concerned only with a small section of the population. The sales figures perhaps cover only the persons employing medicinal worm expulsion measures. All the statistics', however, give a fairly uniform picture which justifies the acceptance of each method of statistical analysis. It is obvious that in actual fact an average of about a fifth of Finland's population is infested with parasites. One individual may be infested several times although the worm is definitely expelled on each occasion. There has probably been some improvement in the situation of late, but much enlightenment work is needed, above all to change the methods of preparing the food, before we can expect this national disease to start disappearing.

SUMMARY

Diphyllobothriasis is a very common infectious disease in Finland. According to various statistics about a fifth of the whole population is infected. The incidence is highest in lake districts, but also in some other areas, where fish consuming habits are prevalent the infestation rate is considerable. In some communes already in the school-age the infestation rate is 15 per cent, and

rises with the age up to about 70 per cent. While the infection is dependent on fish consumption the chief method for preventive measures has been enlightenment work for better food preparation. There has probably been some improvement in the situation after the war, especially in less infected areas, but much work is needed before the situation will be satisfactory.

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FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF TURKU

EFFECT OF 5-HYDROXYTRYPTAMINE ON THE NUCLEUS SUPRAOPTICUS

AND OBSERVATIONS ON ITS EFFECT ON THE NEUROSECRETORY
MATERIAL IN THE MEDIAN EMINENCE; QUANTITATIVE STUDIES

by

E. KIVALO¹ and U. K. RINNE

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In their earlier investigations the writers found that subcutaneously administered 5-hydroxytryptamine (5-HT; serotonin) causes depletion of the neurosecretory material (N.S.M.) in the posterior lobe of the hypophysis (7) and in the cells of the nucleus supraopticus (N.S.O.) and nucleus paraventricularis (N.P.V.) of the rat (8). It has been concluded from these results that the anti-diuretic effect of 5-HT would be due, in part at least, also to the increased release of antidiuretic hormone. This opinion has further been supported by our observation that 5-HT produces a histochemically observable increase of the acetylcholinesterase and acid phosphatase activity in the cells of the magnocellular hypothalamic nuclei (9).

It has been shown that 5-HT causes ACTH release in the anterior lobe of the hypophysis (*e.g.* 6). On the other hand the possibility has been suggested that the N.S.M. going into the portal vessels in the region of the median eminence would be the transmitting substance causing the release of ACTH (13).

In the present investigation we have carried on the study of the effect of 5-HT on the hypothalamus-posterior pituitary-neuro-

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secretory system by investigating histologically its effect on the N.S.M. contained in the supraoptico-hypophyseal tract and by performing quantitative measurements of the nucleolus and nucleus volumes in the cells of the N.S.O. Moreover, the effect of 5-HT on the N.S.M. going into the portal vessels in the region of the median eminence has been investigated.

MATERIAL AND METHODS

White male rats weighing about 180—220 g, altogether 20 animals, were used for this investigation, half of them constituting a control group. Ten experimental animals were injected subcutaneously with 5-hydroxytryptamine sulf.c. creatinine sulf. during seven days in a daily dosage of 0.5 mg per kg body weight (0.1 ml per rat). The 5-HT was together with creatinine as a double salt, dissolved in a solution containing: Natr. sulfis 3 mg, Natr. merthiolat. 0.4 mg, Natr. chlorid. 10 mg, and Aq. dest. steril q.s. ad 2 ml. The same number of animals were injected subcutaneously during seven days with 0.1 ml per rat, daily, of the same solvent. The animals were given food and water *ad lib.* and they were treated in the same manner as the other laboratory animals.

The animals were killed by rapid decapitation 12 hours after the last injection. The hypothalamus was immediately excised and fixed in Bouin's solution for seven days. The specimens were treated in the usual manner and embedded in paraffin. Half of the hypothalami of the test animals as well as the controls were sliced in the frontal plane into cuts of 5 micra and stained with methyl green-pyronin for the nucleus and nucleolus measurements. The other half were sliced in the sagittal plane into cuts of 5 micra and the N.S.M. in them was stained according to Gomori's aldehyde-fuchsin method preceded by KMnO₄ oxydation.

The nuclear diameters were measured with the ocular micrometer at a magnification of 1600 x. Only such nuclei were measured in which the nucleoli were in the middle of the nucleus. The smallest and largest diameter of the nuclei was measured, while only one diameter of the nucleoli was recorded. From the supraoptic nucleus of every animal 100 cells were measured. The nucleus and nucleolus volumes were calculated in the usual manner from the values obtained.

RESULTS

From Table 1 the nucleus and nucleolus volumes of the test animals as well as the controls can be seen. The test animals had a greater nucleus and nucleolus volume than the controls although the difference is statistically significant only in regard to the nucleus volume.

TABLE 1

THE NUCLEUS AND NUCLEOLUS VOLUMES IN CUBIC MICRON IN THE NUCLEUS SUPRAOPTICUS OF CONTROL AND 5-HT-TREATED RATS. THE VALUES OF P ARE RELATED TO CONTROLS

Group	Number of Cells Counted	Nucleus μ^3	P	Nucleolus μ^3	P
Control	500	399 \pm 12		9.9 \pm 1.0	
5-HT-treatment	500	462 \pm 35	< 0.05	10.9 \pm 0.7	> 0.05

On examining the quantity of N.S.M. in the supraopticohypophyseal tract it was found to be distinctly less in the test animals treated with 5-HT than in the controls. (Fig. 1, 2).

In the median eminence around the portal vessels and in the transversal nerve fibres running to them the quantity of N.S.M. was found to be normal in the controls, as has been observed previously, whereas among the test animals subjected to 5-HT treatment some displayed more N.S.M. than the controls, particularly around the portal vessels and within them, all other test animals having a quantity at least equalling that in the controls (Fig. 3, 4). Attention was particularly drawn to the fact that within and around the portal vessels and in the transversal fibres running to them the quantity of N.S.M. as related to the N.S.M. in the supraopticohypophyseal tract, was clearly greater with the 5-HT treated animals than with the controls.



Fig. 1.

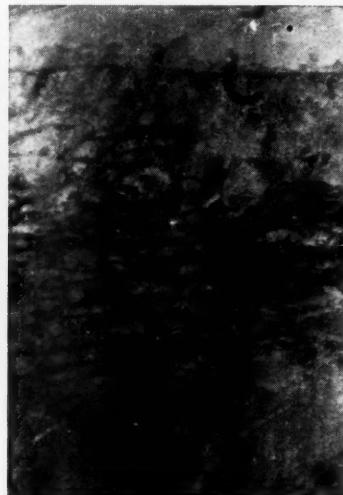


Fig. 2.

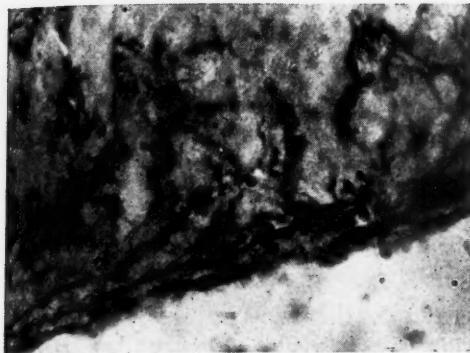


Fig. 3.



Fig. 4.

Fig. 1. — Tractus supraoptico-hypophyseus in the median eminence of a control rat. There is visible the normal quantity of neurosecretory material and Herring-bodies. Aldehyde-fuchsin $\times 960$.

Fig. 2. — Tractus supraoptico-hypophyseus in the median eminence of a 5-hydroxytryptamine-treated rat. The neurosecretory material is clearly decreased compared to controls. Aldehyde-fuchsin $\times 960$.

Fig. 3. — Neurosecretory material around and in the portal vessels in the median eminence of a control rat. Aldehyde-fuchsin $\times 960$.

Fig. 4. — Neurosecretory material around and in the portal vessels in the median eminence of a 5-hydroxytryptamine-treated rat. Aldehyde-fuchsin $\times 960$.

DISCUSSION

The antidiuretic effect of 5-HT has usually been attributed to the peripheral, renal effect of this drug (4, 12). However, Abrahams and Pickford (1) have suggested the possibility that the antidiuretic effect of 5-HT would at least in part be due to the release of antidiuretic hormone. The authors have shown in their histological and histochemical investigations that 5-HT causes in the rat depletion of the N.S.M. in the posterior lobe of the hypophysis and in the cells of the N.S.O. and N.P.V. and increases the acetylcholinesterase and acid phosphatase activity of these cells (7, 8, 9). The results obtained in the present investigation show further that 5-HT stimulates the hypothalamus-posterior pituitary-neurosecretory system, causing depletion of the N.S.M. in the supraoptico-hypophyseal tract and increasing the nucleus and nucleolus volumes of the N.S.O. Nucleus and nucleolus measurements were made on the N.S.O. only because it has been shown that in the cells of the N.S.O. and the N.P.V. these increase linearly in states in which the hypothalamus-posterior pituitary system is stimulated *e.g.* by dehydration (11, 3, 10, 2). The results now obtained, and on the other hand our earlier result, show that 5-HT treatment stimulates the hypothalamus-posterior pituitary neurosecretory system. However, it has to be taken into consideration as one of the possibilities that the administration of a pharmacological substance produces a non-specific, though slight, stress (*e.g.* 6), which also causes activation of the hypothalamus-posterior pituitary system, as could be shown in previous investigations (13). On the other hand the 5-HT with creatinine as a double salt dosis employed in this work is 0.5 mg per kg and according to Erspamer the acute toxic dosis of the 5-HT for rats on subcutaneous administration is 117 mg per kg, while the chronic administration of 1 mg daily as subcutaneous injections has not caused any general symptoms or any macroscopic or microscopic changes in the internal organs during 30 to 100 days (5). It is therefore likely that the stimulating effect of 5-HT upon the hypothalamus-posterior pituitary system is not due to any stress effect but is rather a primary effect. For this reason it is suggested that the antidiuretic effect of 5-HT is due, in part at least, to the increased release of antidiuretic hormone in addition to the renal effect.

It has been shown in earlier investigation that 5-HT causes ACTH release from the anterior lobe of the hypophysis (*e.g.* 6). In the present investigation we noted with some animals a positive correlation between the N.S.M. going into the portal vessels and the increased ACTH release. Therefore, it is suggested that the N.S.M. going into the portal vessels would have some association with the release of ACTH, a possibility that has been pointed out in earlier investigation as well (13).

SUMMARY

The effect of 5-hydroxytryptamine (5-HT; serotonin) on the nucleus and nucleolus volumes in the nucleus supraopticus (N.S.O.) of the hypothalamus and on the neurosecretory material (N.S.M.) going into the portal vessels in the region of the median eminence as well as on that contained in the supraoptico-hypophyseal tract in the rat has been investigated. It was found that 5-HT produces an increase of the nucleus and nucleolus volume in the N.S.O. On the other hand the supraoptico-hypophyseal tract contained distinctly less N.S.M. with the 5-HT treated animals than with the controls, whereas the quantity of N.S.M. going into the portal vessels in the region of the median eminence was equal in magnitude, as compared with the controls, or even greater in some test animals. The mechanisms of effect of 5-HT on the hypothalamus-posterior pituitary system have been discussed, and it has been concluded that the antidiuretic effect of 5-HT is due, in part at least, to the increased release of antidiuretic hormone caused by this drug in addition to its renal effect. On the other hand it is thought on the basis of the results obtained that the N.S.M. going into the portal vessels may possibly be associated with the release of ACTH.

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FETAL INTESTINAL ABSORPTION AND TRANS-
PLACENTAL CARRIAGE OF PHENOLPHTHALEIN IN
GUINEA PIG

by

P. PÖRSTI, A. SALMIVALLI and K. HARTIALA¹

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The transfer of certain substances in the organism requires special measures in order to keep the substances in such physicochemical form that the penetration through cellular membranes is possible.

Thus it is known that the active transport of certain steroids and phenols is associated with conjugation procedures which keep these substances in a soluble form.

Our recent work has dealt with these conjugation processes such as they occur in the gastrointestinal tract and in the liver (2, 3, 4, 5, 6, 7, 8, 9, 10, 11). In connection of these studies no demonstrable amount of glucuronide conjugation has been detected in the placenta or its components in vitro conditions (2, 3). Information of the material exchange of various substances between the fetus and mother is of considerable importance from the point of view of nutritional and regulative functions during the fetal development.

The present study was undertaken in order to obtain information of the active glucuronide conjugation of the fetus in utero.

Also information was sought of the transport through the placenta both from the mother side to the fetus and vice versa from the fetus to the mother of a such substance which is known to be conjugated as a glucuronide and excreted from the organism in this form.

¹This study has been supported by a grant from the Sigrid Jusélius Foundation.

MATERIALS AND METHODS

Guinea pigs were used in this study. This animal was found to be suitable for the purpose. It has been shown that both the intestinal and hepatic glucuronide conjugation processes occur in this animal both in adult and fetus (1, 7). The fetus of this animal are relatively large and suitable for operative and chemical manipulations. Altogether 47 pregnant guinea pigs were used. The animals were studied usually during the last days before the expected parturition. Also some studies were made during the earlier phases of pregnancy. The studies were performed in two ways.

Part 1. In the first part (including 30 pregnant guinea pigs) one fetus in each animal was exposed through a mid-line abdominal excision — under nembutal anesthesia — and by opening the uterine wall at the place nearest to the mouth of the fetus.

After puncturing the membranes a thin plastic tube was introduced into the stomach of this fetus. Phenolphthalein phosphate (250 mg/2 ml) was then injected into the stomach, the mouth of the fetus was kept closed with a silk string fastened around it. The uterine and abdominal walls were closed with silk sutures. The size of the fetus ranged between 10—14 cm and the weight from 40—100 g. After 3—4 hours the uterus was exposed again and blood samples were taken from the treated fetus, from the untreated fetus in the same animal and from the mother. The blood samples were centrifuged and both the free and conjugated phenolphthalein were determined.

Part 2. In the second part of the experiments (17 animals) a phenolphthalein compound was injected into the uterine artery of the adult guinea pig. In the previous case the abdominal wall was opened with a mid-line excision. From 5 minutes to 4 hours after the injection blood samples were taken both from the mother and fetus. The phenolphthalein determinations from the plasma were made according to the following same procedures.

Detection of Free and Conjugated Phenolphthalein. — The present method is a modification of that used by Talalay, Fishman and Huggins (12). The blood samples which were most easily obtained by a heart puncture were treated with 3.8 per cent sodium citrate and centrifuged. Into 0.1 ml of plasma was added 0.8 ml of a acetate

buffer solution (pH 4.5) and in each other sample 0.1 ml of a β -glucuronidase solution containing 24 units of the enzyme (Worthington). The controls received 0.1 ml of the acetate buffer. The test tubes were incubated in a water bath at 37°C for 24 hours. The proteins were precipitated by adding 1 ml of 10 per cent trichloroacetic acid and by centrifugation. The clear supernatants were made alkaline by adding a mixture of glycine and sodium hydroxide buffer (pH 10.45). In presence of free phenolphthalein the typical colour was developed and measured with a Beckman spectrophotometer using the wavelength 550 m μ . This reading gives the value for the amount of phenolphthalein liberated by the β -glucuronidase hydrolysis and indicates thus the amount of conjugated phenolphthalein when subtracted from the control values (without the β -glucuronidase solution).

The protein precipitate carries the most of free phenolphthalein present in the plasma. This can be detected by adding the alkaline glycine buffer to the precipitate. The presence of free phenolphthalein is indicated by a strong colour formation. In this case the turbidity of the sample does not allow exact quantitative information; these preliminary results are therefore taken only as a qualitative demonstration for the presence of free phenolphthalein in these specimens.

The liver phenolphthalein determinations were made from homogenates ground with a Potter-Elvehjem homogenator and which were then treated as the blood samples.

The urinary determinations were made as above but without the protein precipitation procedures.

RESULTS AND DISCUSSION

Fetal Absorption of Phenolphthalein Phosphate. — In all of the cases it was found that the intrauterine feeding of the phenolphthalein phosphate to the fetus was followed by the appearance of phenolphthalein in the fetal blood. These results are illustrated by fig. 1. The blood concentrations and also corresponding extracts made from the liver of the same fetus showed a markedly high concentration of phenolphthalein. Treatment of the blood sample with β -glucuronidase resulted in a great increase in the free phenolphthalein values which indicates that part of the phenolphthalein is in the form of a glucuronide.

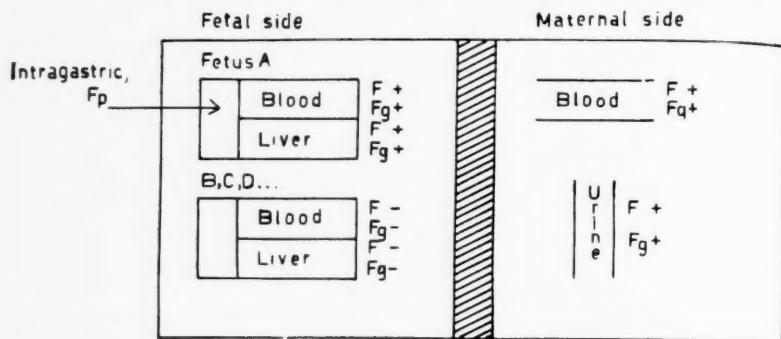


Fig. 1. — Studies of the intrauterine gastric feeding of 250 mg of phenolphthalein phosphate (Fp) in the guinea pig.

F, free phenolphthalein,
Fg, phenolphthalein glucuronide.

On the other hand no phenolphthalein could be detected in the blood or liver of those intact fetus which were present in the same uterus but which had not received the intragastric feeding of phenolphthalein.

The blood and urine of the mother contained phenolphthalein after the intragastric fetal feeding. In the urine phenolphthalein appeared some $\frac{1}{2}$ —1 hour after the fetal treatment. Treatment of the blood or urine samples with β -glucuronidase and approximate calculations indicate that most of the phenolphthalein is in the conjugated form. Occasionally the maternal blood samples showed no phenolphthalein. In these cases, however, the urine contained exceptionally high amounts of the same substance which indicates a quantitative excretion and elimination of it in the urine.

Few studies were also made in which pure phenolphthalein was used instead of the phosphate. In these cases it was found to be precipitated in the fetal stomach. In these cases the fetal blood was always negative for phenolphthalein.

Administration of Phenolphthalein Phosphate, Phenolphthalein, or Phenolphthalein-glucuronide into Uterine Artery. — These experiments were performed on 17 pregnant guinea pigs. In no case was the administration of phenolphthalein phosphate (Fig. 2) in to the uterine artery followed by the appearance of phenolphthalein in the fetal side of placenta. Thus no signs of phenolphthalein, either free or as a glucuronide could be detected in the fetal blood, liver or amniotic

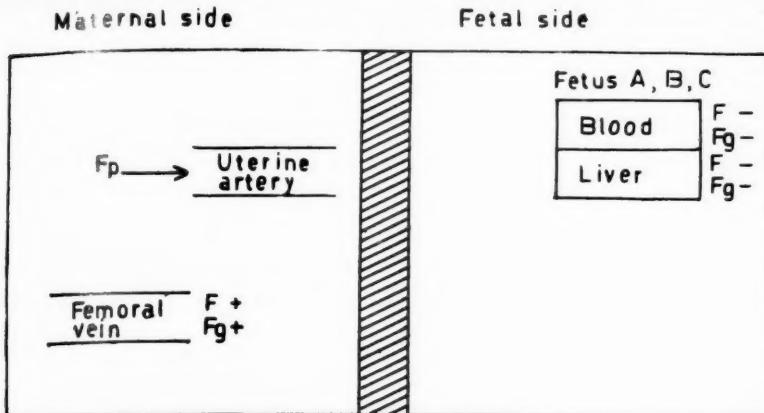


Fig. 2. — Placental transfer of phenolphthalein phosphate (25 mg) administered into the uterine artery in the guinea pig.

Fp, phenolphthalein phosphate

F, free phenolphthalein

Fg, phenolphthalein glucuronide

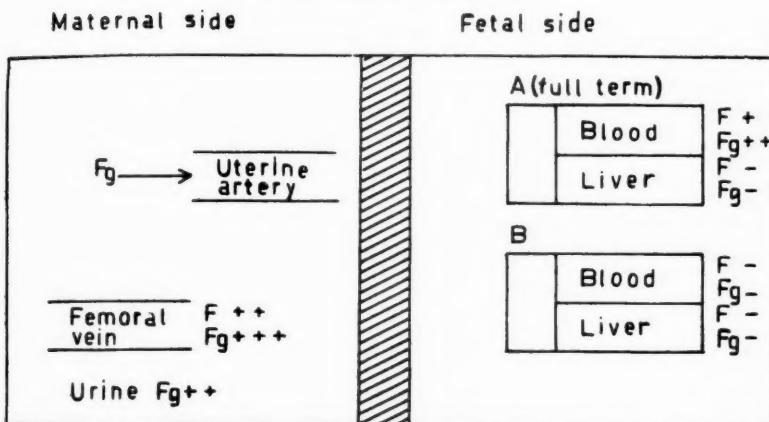


Fig. 3. — Placental transfer of phenolphthalein glucuronide administered into the uterine artery in the guinea pig.

F, free phenolphthalein

Fg, phenolphthalein glucuronide

fluid. The blood of the mother contained phenolphthalein also in a conjugated form as a glucuronide after the injection of the phenolphthalein phosphate.

Pure phenolphthalein when administered into the uterine artery could not be detected in the mothers systemic blood circulation.

Similar experiments were then performed with phenolphthalein glucuronide (Fig. 3). The placenta and fetus were exposed after 5 minutes and kept alive another 30 minutes. In these experiments the fetal blood constantly contained phenolphthalein, the mean concentration being remarkably high.

The hepatic tissue, however, did not contain phenolphthalein.

In these same experiments the remaining fetus were removed after 2 hours. At this time no phenolphthalein was detected in the fetal blood or liver, regardless of the fact that the mother had a rather high phenolphthalein concentration in her blood. Few additional experiments were also performed in the earlier phases of the pregnancy. It appeared in the smaller fetus that the transfer of phenolphthalein glucuronide from the maternal side to the fetus was diminished so that in fetus under the length of 10 cm no phenolphthalein glucuronide could be detected anymore in the fetal side.

These observations indicate that the conjugation processes in nearly full-termed fetus are really functioning and that substances undergoing these processes can be absorbed in this form from the gastrointestinal tract of the fetus. These observations provide further support for the previous studies *in vitro* made with tissue slices which show that the fetal tissues are capable of performing active glucuronide conjugation (7, 1). According to Dutton (1) the fetal stomach shows in this respect even greater capacity than the liver.

The present observations even though only qualitative indicate that the transport across the placenta of such substance which otherwise undergo a conjugation is not entirely independent of the form in which they are introduced into the organism. Thus free phenolphthalein applied to the uterine artery is not transferred to the fetal side. When the same substance was administered as a glucuronide it could easily be detected in the fetal side. These observations may be taken as an indication that these conjugations serve the purpose of facilitating the transfer of certain substances in the blood and through cellular carriers. In the intestine they would serve the purpose of active absorption (4, 6).

SUMMARY

Intragastric administration of phenolphthalein phosphate in the nearly full-term guinea pig fetus in utero resulted in the appearance of easily detectable amounts of phenolphthalein glucuronide both in the fetal blood and maternal blood and urine. This indicates an active fetal glucuronide synthesis.

Administration of different phenolphthalein compounds into the uterine artery in pregnant guinea pigs indicated that this compound was transferred into the fetal side preferably when applied as a glucuronide conjugate.

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LOCAL CHANGES IN EXPERIMENTAL ARTHRITIS AND THE EFFECT ON THEM OF HYALURONIDASE

by

L. LÖFGREN¹

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About ten years ago, Selye's (26) research team succeeded in working out a method for inducing experimental arthritis. New possibilities were thereby opened for research into this field — of such topical interest in several medical disciplines. By injecting a diluted formaldehyde solution just beneath plantar aponeurosis of the hind paw of white rats, they caused intensive hyperemia and edema in the whole paw in the course of a few minutes. About ten days later, when the acute reaction had abated, a more chronic type of joint affection arose; proliferation of connective tissue in the region of the ankle-joint occurred. These changes were manifested, particularly in the test animals given several formaldehyde injections at intervals of one or two days.

As is well known, the commonest arthritides in man, those of partly unclear etiology and genesis are divided into: 1) arthritis of rheumatic fever, 2) rheumatoid arthritis (syn. atrophic arthritis, chronic infectious arthritis, arthritis deformans), and 3) osteo-arthritis (syn. hypertrophic arthritis, degenerative arthritis, osteo-arthrosis).

Against the background of the results of various experiments regarding arthritides, Selye's (27) statement appears to be warranted. He says that it hardly seems just to rigorously classify different kinds of arthritis with more or less unknown etiology, in which the differences often are difficult to distinguish whereas

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the similarities are striking. — With this in mind, I have endeavoured to gain experience of Selye's method and to discover the changes occurring after experiments over a rather long period. Animal hyaluronidase was used in the tests, with a view to the intensive formation of edema which, according to many investigators (1, 26) is associated with acute formaldehyde-arthritis, and also with the conception »collagenosis», first introduced by Klemperer (18) — later modified and given the collective denomination diseases of the connective tissue — but excluding study of the histo-chemical pathogenesis of experimental arthritis.

Hyaluronidase. — In 1928, Durand-Reynals (12) reported how intracutaneous injection of testicle extracts accelerated the distribution of simultaneously injected dyes, various viruses and toxins. This discovery gave rise to the term »spreading factors». Six years later, Meyer and Palmer (23) succeeded in isolating certain substances from vitreous body of cattle and called them hyaluronic acids. These polysaccharide acids which are combined with proteins and constitute an important part of the ground substance of connective tissue, occur in most of the mesenchymal tissues and also, for instance in the fluid of joints. In 1936, Meyer and co-workers (22) reported their observation of pneumococci having the capacity to dissolve certain polysaccharides present in the tissues. Three years later, Chain and Duthie (7) discovered a spreading factor capable of splitting hyaluronic acid. By hydrolysis of hyaluronic acid, this enzyme, called hyaluronidase, reduces the viscosity of the ground substance of connective tissue which is caused by the hyaluronic acid. — It has not been satisfactorily clarified where this enzyme is formed; but it can be obtained both from the testicle of Mammalia and from snake poison, for instance, and is also found in several bacteria. This explains the denomination animal hyaluronidase as distinct from microbe hyaluronidase. — Hyaluronidase is considered to act, for instance, on the capillary permeability (8) and capillary fragility (27), the rapidity of bacterial invasion (11), malignancy of tumours (10), peritonites and peritoneal formation of adhesions (9), and, further, to play a role in the arisal of polyarthritic joint exudates (17) and in the pathogenesis of so-called diffuse collagen-diseases (6, 27). — In recent years, alongside with comprehensive experimental investigations — hyaluronidase has been used for therapeutic purposes in many branches of clinical medicine: for instance, for subcutaneous and intramuscular injections and infusions to accelerate resorption and spreading (14, 28), and for local anesthesia (14, 21, 28). Moreover, it has been successfully used in treatment of, and for prophylactic purposes in cases of postoperative and traumatic edema and hematoma (21, 28), and in treatment of bursitis, traumatic and idiopathic hydrarthrosis (13), and hemarthrosis (21). — The local action of hyaluronidase has been observed to cover a period of about 24 hours (21).

EXPERIMENTAL TECHNIQUE

The animals used in the present experiments were 46 adult albino rats, of which 10 served as controls. At the time of starting the tests, their weights varied between 70 and 110 grammes and on termination, six and a half months later, between 175 and 320 grammes.

A 1 per cent solution of formaldehyde and a standardized testicle hyaluronidase preparation (Invasin¹) was used. —

As a pre-test, two rats were given 2 ml of formaldehyde solution without anesthesia, just beneath plantar aponeurosis of the right hind paw, and one was given the injection in the region of the right knee on two consecutive days, and the reaction was studied. In the same manner, three other rats were given 2.5 V.R.E. (1 V.R.E. = viscosity reduction unity = the amount that under stated conditions, reduces the viscosity of a hyaluronic acid solution by half during the course of 10 minutes) into the hind paw on four consecutive days.

Twenty of the test animals were given injections of 2 ml of formaldehyde solution, as described, into the right hind paw. The injection was repeated four times at intervals of four days, and followed by five injections at intervals of one month. One or two rats were decapitated on the next occasion, before the injection was given. Ten rats were first given 2.5 V.R.E. of hyaluronidase and ten minutes later 2 ml of formaldehyde solution, as described, into the same part, and at the same intervals as those treated only with formaldehyde. One animal was decapitated in connection with each of the following injections. Ten animals serving as controls were given 2 ml of saline, the same technique being employed. — Four of those treated only with formaldehyde and one of the controls died during the course of the experiments.

Observations were made after each injection regarding the animal's pattern of behaviour and the reaction in the injected paw. By means of a gliding compasse, measurings were made in transverse direction, at the height of the ankle-joint and immediately beneath it.

The test animals were kept isolated in cages, each test group separately, in a stable with a fairly constant temperature; all were fed on the same diet.

¹ My thanks are due to Messrs. H. Lundbeck & Co. A/S, Copenhagen, for supplying the preparation Invasin for these experiments.

After decapitation, the right hind leg was removed, and the region of the ankle-joint was divided into two equal parts in sagittal direction. A portion of the periarticular soft tissue was fixed in a fresh 4 per cent aqueous lead acetate solution for 48 hours; the remainder was fixed in formol-alcohol. Decalcification of the bone was done in 5 per cent trichloro-acetic acid. The following staining methods were used: Delafield's hematoxylin-eosin, Weigert's hematoxylin-van Gieson, periodic-acid-Schiff (P.A.S.), Weigert's fibrin staining and aqueous toluidine blue staining. A series of soft part tissue was stained with toluidine blue, after having been exposed to the action of hyaluronidase in a thermostat at 37° C for 18 hours.

PRE-TEST

The pre-tests revealed that the reaction of the rats given injections of formaldehyde solution beneath plantar aponeurosis was distinctly acute, while the injection given in the region of the knee caused only slight swelling at the site of injection. After repeated injections of hyaluronidase the animals were quite unaffected and there was no reaction in the paw; swelling due to the injected fluid only occurred.

TESTS WITH FORMALDEHYDE

The animals given a one per cent formaldehyde solution, as previously described, reacted within a few minutes after each injection by considerable swelling and the whole paw was highly flushed. The rat carried its paw, licking it frequently. There was general animosity and irritability which lasted for about an hour. Gradually, they calmed down, started eating, and making attempts to use the previously so stubbornly protected paw. On the fourth day, when the rats were given their second injection, there was still swelling, especially in the metatarsal region, and also slight swelling in the tarsus-ankle-joint. When the animals were given an injection every fourth day, a total of four injections, it appeared that the swelling did not abate after the later injections as much as after the first ones, but simultaneously with the swelling of the ankle-joint increasing after the injections, the changes assumed the appearance more of chronic indurative arthritis. The intervals between the injections were now prolonged to one month, and the

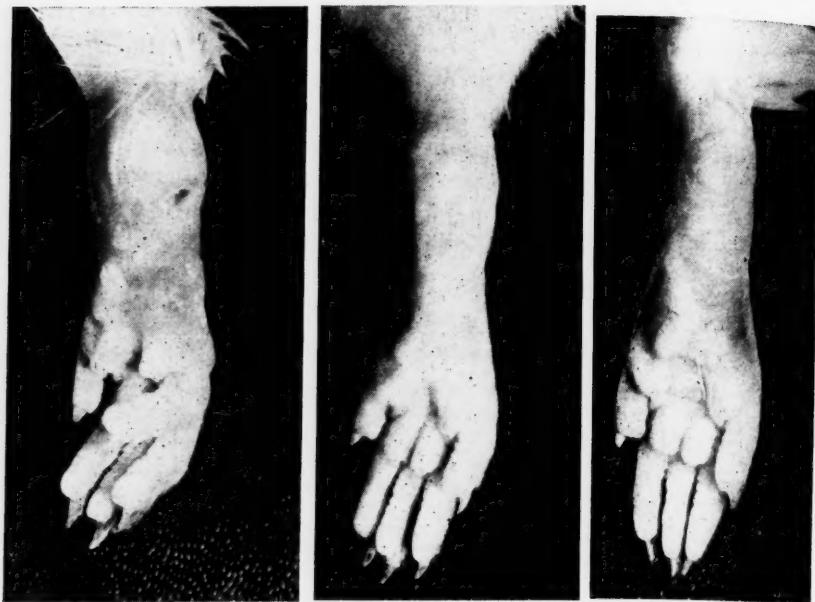


Fig. 1. — Middle: hind paw of control rat. Left: hind paw of rat one month after last of eight injections of 1% formaldehyde solution beneath plantar aponeurosis. Intense indurative swelling round ankle-joint and small cicatrice remaining after healed skin necrosis. Right: hind paw of rat given hyaluronidase and formaldehyde. Photograph, like previous one, taken one month after eight injection. Swelling greatly diminished and skin puckered, a sign of the swelling having receded.

rats were given five further injections, thus a total of nine injections. During the interval, the swelling did not recede but, especially the induration setting in round the ankle-joint assumed a »self-maintaining», chronic character. This is distinctly seen in Figure 1, which shows the paw of a rat a month after the eighth injection, and next to it the control. — To shed some light on the degree of swelling of the paw, it may be mentioned that the transversal diameter immediately distal of the ankle-joint, and also at the height of it, in the controls, was about 5–6 mm, whereas the former measure noted the day after the third injection had risen by one third, to 8 mm on an average, while the latter measure was 6 mm on an average, thus being the same as in the controls. A day after the fourth injection, the measures were 8.5 and 7 mm, on an average. A month later the swelling had receded somewhat, the measure distally of the joint being 7 mm and at the height

of the joint 6 mm. From now on, the swelling of the ankle-joint increased comparatively more, showing the same diameter as the part of the paw immediately distal of the ankle-joint, both measures varying between 7 and 8 mm. — After the second injection there arose, in two rats, necrosis of the skin at the site of the injection; there were no abscess formations. Similar observations were made with regard to some other rats after the later injections. The necroses healed during the free intervals, superficial cicatrices being formed.

TESTS WITH HYALURONIDASE AND FORMALDEHYDE

The entire paw of the test animals given hyaluronidase and formaldehyde solution was swollen and red after the first injection of formaldehyde, similarly as in those given formaldehyde alone. The animals were unsettled and ferocious, but calmed down considerably more rapidly than did those given formaldehyde alone. Many of the rats calmed down as soon as 10—15 minutes after the injection, and 20—30 minutes after the injection the intense flush of the paw seemed to decrease. However, on the following day, the paw was still somewhat swollen, but the animals appeared to move about easily, using the injected paw without difficulty. After the second injection, four days later, the same course was noted, and on the following day the transversal diameter immediate distal of the ankle-joint was slightly larger than that in the controls, but at the joint it was of the same size as in the controls. No skin necroses were seen. At the time of the fourth injection of hyaluronidase and formaldehyde, the paw was swollen: 7 mm in diameter distally of the joint and 6.5 mm at the site of the joint. Four of the animals presented quite small skin necroses in planta at the site of injection. After the fourth injection, when the interval had been prolonged to one month, the swelling of the paw of these test animals abated gradually during the course of a week; the diameter measured at both points was 6.5 mm on an average; another week later it had been reduced to almost the same as in the controls. A shift in the swelling towards the ankle-joint was however also observed in these animals. After the following injections the process was similar to that already described in this connection. At the end of a month, after each

injection, the swelling was markedly less than in the animals treated with formaldehyde alone. It was distinctly seen how the skin, previously shiny and extended due to swelling, was now puckered up. Moreover, the ankle-joint did not take on a chronic induratively swollen appearance as in the rats given formaldehyde alone. Figure 1 shows distinctly the difference between the animals treated in the different ways, and the controls. The skin necroses were less numerous and smaller in circumference in the rats given hyaluronidase and formaldehyde compared with those receiving formaldehyde alone.

HISTOLOGICAL FINDINGS

Rats Treated with Formaldehyde Solution. — As soon as after the first injections, the animals treated with formaldehyde alone, showed a number of rather small necrobiotic areas in the cutaneous connective tissue. It was edematous and the edema reached as deep as to the periarticular tissue. Round cells were accumulated round the necrobioses, perivascularly and diffusely. Small numbers of leukocytes were counted as well. Sometimes round cells were especially numerous in loose periarticular-peritendinous connective tissue where also exudate occurred. At an early stage, signs of a change in activity were noted in the connective tissue surrounding the necrobioses. At the same time as the connective tissue assumed a more homogenized swollen appearance, numerous large cells with blister-like nuclei, sometimes with two or more nuclei in the same cell, small giant cells, were seen. The impression was that the cells were swollen fibroblasts, forming granulomata-like shapes. In some parts, these changes were particularly widespread extending towards the border of stratum papillare. After the later injections, the changes in the cutaneous and periarticular connective tissue were considerable, the same as those just described, but differing, however, in that more sclerosed cicatricial, dense connective tissue, poor in cells, was seen, and, in parts, in the depth there were some small nodules, a collection of epithelioid cells. On staining by van Gieson's method, some yellowish bands were seen and, in large areas, the connective tissue was slightly yellow suggesting fibrinoid degeneration (5). This is — simultaneously considering the morphological changes in the connective

tissue — to some extent supported by the results of the fibrin staining when extensive areas of connective tissue stained a vivid violet.

In general, the vessels in the connective tissue were not noticeably filled with blood. This is understandable as the examination was not as a rule performed until the acute congestion had receded and edema only remained.

The mast cell count in the connective tissue varied, the cells were irregularly disturbed and at different depths, most of them,

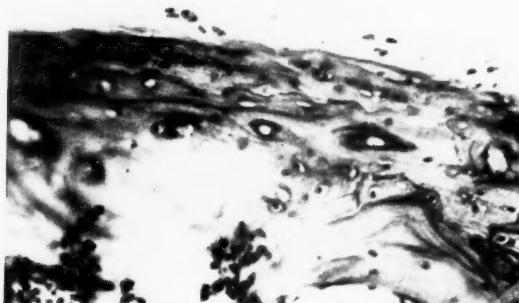


Fig. 2. — Photomicrograph taken one month after sixth formaldehyde injection; ossification extends toward uneven, fibrincoated surface of joint cartilage. Hematoxylin-eosin. $\times 170$.

however, occurring at a certain distance from stratum papillare, sometimes in large aggregations, sometimes less numerously. The necrobitoc areas contained no mast cells. In different preparations and in different parts there were cells partly with larger and partly with smaller granules, partly with more granules partly with less, hence the stainability varied. Disrupted cells and granules lying free in the tissue were also seen. After pre-treatment with hyaluronidase of some preparations, and on staining with toluidine blue, there occurred no divergencies from the controls.

Changes in the cartilage and bone of the ankle-joint were first observed in the preparations made one month after the sixth injection. In some instances fibrinous coatings had been previously noted in the joint, however. The ossification extended toward the surface of the joint cartilage (Fig. 2), and conglomerations of the cells in the perpendicular-transitional zone of the joint cartilage

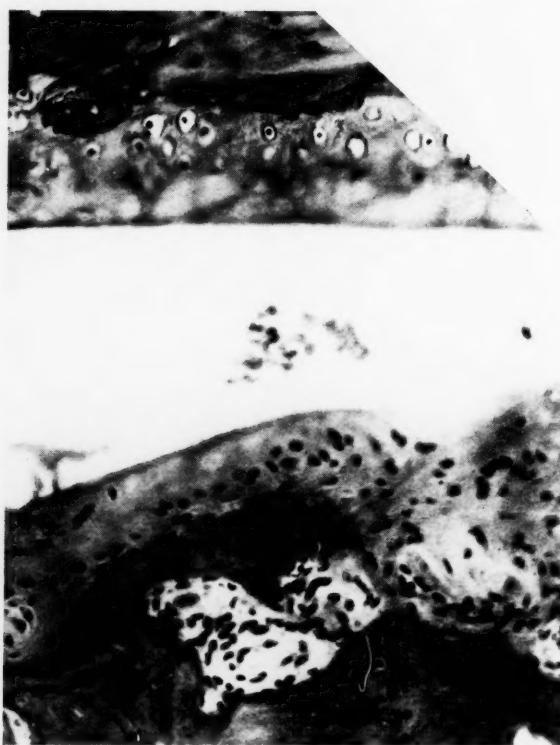


Fig. 3. — Photomicrograph taken one month after seventh formaldehyde injection. Ossification extends more toward uneven fibrin-coated surface of joint cartilage. Bone marrow next to cartilage is substituted by connective tissue. — Fixed in formalin-alcohol. Hematoxylin-eosin. $\times 170$.

occurred. After the seventh injection, the ossification was seen in some parts to extend more and more toward the surface of the joint cartilage and the bone marrow closest to the cartilage was substituted by connective tissue (Fig. 3). After the eighth injection, five and a half months after starting the experiments, a defect in the joint cartilage was seen which was surrounded by cartilage cell proliferation, denoting a vital, reparative reaction, whereas the cells farther from the defect had largely lost their staining capacity (Fig. 4). The surface of the cartilage was uneven in several parts and covered with fibrin.

Epidermis also underwent changes. Keratosis-hyperkeratosis increased, assuming a squamous character as the tests progressed.

Epidermis was more or less creased and stratum papillare more or less marked, depending on the degree of swelling of the paw. Occasionally there was intense edema immediately below epidermis which, toward the end of the tests, in addition to stratum corneum, consisted in some parts only of two or three cell layers, composing their stratum germinativum. The edema and induration caused atrophy of the skin, due to increased tension, which is revealed



Fig. 4. — Photograph taken after eighth injection of formaldehyde, showing defect in joint cartilage, surrounded by proliferation of cartilage cells close to defect, while most cartilage cells farther from defect have lost their staining capacity. Fixed in formalin-alcohol. Hematoxylin-eosin. $\times 170$.

also by its glossiness and scaliness. — The skin necroses were typical: a somewhat withdrawn necrotic mass with fibrinoid degenerated ground surrounded by a wall of inflammatory cells and granulation tissue.

Rats Treated with Hyaluronidase and Formaldehyde. — The rats treated with hyaluronidase and formaldehyde presented similar histological changes on the whole as did those given formaldehyde alone. But the changes in the former did not appear to be so extensive as in the latter. A month after the fifth injection of hyaluronidase and formaldehyde, fibrin coagula containing synovial

endothels were seen in the ankle-joint. The periarticular tissue-synovial membranes were edematous and the capillaries filled with blood. Slight edema in the cutaneous connective tissue and a conglomeration of large cells with blister-like nuclei were also noted. At later stages, there were signs of progressing ossification of the joint cartilage, similar to that described in the rats treated with formaldehyde alone.

To summarize, it may be said that the changes occurring in the rats treated with formaldehyde alone and those given hyaluronidase and formaldehyde were histologically similar, and differed only as to degree of intensity. *When the tests were prolonged over a rather long period, certain changes in cartilage and bone occurred.* With the use of hyaluronidase, the external chronic indurative changes in experimental arthritis may be counteracted to a considerable degree, and their course seems to be similar to that of periarticular histopathological changes. -- The experimentally induced changes have a striking similarity to some of the arthritides in human medicine and may thus be of assistance in different kinds of experimental tests.

COMMENTS

After Selye's (26) publication of a method for consistent production of arthritis by injection of a diluted formaldehyde solution beneath plantar aponeurosis in test animals, a number of investigators have employed his method, for instance, for studies of the effect of various therapeutic interferences. Selye's team of research workers had previously observed that the mineralo-corticoids often produced arthritis-periarthritis. This they associated pathogenetically with the shift in the gluco-corticoid/mineralo-corticoid balance. Their assumption was substantiated *inter alia* by the fact that, as early as in 1946, patients with chronic rheumatoid arthritis were favourably affected by adreno-cortical extracts (4), particularly rich in, especially gluco-corticoids, primarily cortisone (16). Further, by the fact that favourable action on arthritis was obtained by the formerly used, empirically founded, non-specific therapeutical methods, and by different kinds of unspecific stressors, acting as alarming stimuli in a general

adaptation-syndrome. The results of a number of experimental tests, and clinical experience substantiate Selye's theories.

When the pathogenesis of experimentally induced arthritis by means of formaldehyde is concerned, the primary sign is inflammation attended by the classical symptoms. The necrobioses serve to maintain the inflammation and its symptoms, *i.e.*, circulatory disorders and edema, form a link in the circulus vitiosus which has arisen. The reaction in the connective tissue is a sign of the proliferative changes associated with inflammations in general. That experimental arthritis is susceptible to hormonal influence has been proved, and the action is probably furthered by hormonal as well as neurogen routes.

Inflammatory edema is of fundamental significance for the origination of indurative sclerosis around the joint; due to this, the effect of hyaluronidase is inhibiting -- as shown in the present experiments.

Although formaldehyde is a stressor with the capacity to mobilize ACTH — corticosteroids which counteract the arisal of chronic indurative changes in the joints and inhibit proliferation of connective tissue in general, severe induration occurs around the ankle-joint after prolonged treatment with formaldehyde, as revealed in the present study. On the other hand, if the animals were simultaneously treated with hyaluronidase, these changes were well counteracted, probably owing to the fact that the edema disappeared before it had had time to become organized. This agrees well with earlier investigations showing that sympathectomy and tetra-aethyl-ammonium-bromide have a protecting effect also in this respect (2, 3), most closely by bringing about improved circulation. — Hyaluronic acid, one of the mucopolysaccharides in the ground substance of connective tissue, is strongly water-binding, and the depolymerizing effect of hyaluronidase upon the acid explains the intervening mechanism of the enzyme.

SUMMARY

Tests were made with Selye's and his co-workers technique to induce formaldehyde arthritides in rats. The experiments were continued over a period of six and a half months. The series comprised 46 test animals, ten of which served as controls. Initially, the rats were given four injections at intervals of four days,

followed by five injections at intervals of one month. Parallelly, tests with hyaluronidase were made to discover its effect on experimental arthritis.

The arthritic changes arising were conspicuously marked by an acute phase after each injection, followed by chronic induration at a later stage. These chronic changes were strikingly counteracted by simultaneous treatment with hyaluronidase. However, the histological picture of the rats treated with formaldehyde alone and those simultaneously given hyaluronidase was similar on the whole; the only difference was in degree of intensity. Towards the end of the experiments, changes in the cartilage and bone of the ankle-joint were observed in both groups. The favourable effect of hyaluronidase was considered due to its action on inflammatory edema.

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THE EFFECT OF RESERPINE PRE-TREATMENT IN EXPERIMENTAL PULMONARY EMBOLISM

by

M. H. FRICK

(Received for publication March 17, 1959)

The pulmonary hypertension following pulmonary embolism is by some investigators considered to be explicable on the base of merely a mechanical obstruction of pulmonary circulation (10, 11, 45). Many investigators, however, regard vasoconstriction in the pulmonary circulation as the cause of the pulmonary hypertension (3, 32, 33, 38, 42). This opinion is supported especially by investigations in which it has been shown that pulmonary embolism produces increased pressure in both the pulmonary artery and the pulmonary vein, while pressure in the left auricle remains unchanged (19, 22, 25, 34).

Daly and Hebb (12) demonstrated with dogs that the vasomotor nerve fibers of the lungs form a part of the cervical vago-sympathetic trunk. Daly and associates (13) also found in perfusion experiments that stimulation of the thoracic sympathetic nerve, ganglion stellatum and the middle cervical ganglion reduced pulmonary circulation by as much as 30 per cent. The addition of ergotoxin to the perfusion blood prevented this reduction. By faradic stimulation of the ganglion stellatum, Leriche (26) produced pulmonary vasoconstriction.

On the other hand, ganglion stellatum block has been found to be beneficial in the treatment of patients with pulmonary embolism. Leriche and associates (27) presumably were the first to pay attention to this therapeutic possibility. Faxon and associates (16), using stellatum block after pulmonary embolism, obtained good results in

three out of four patients. Price and associates (38) and Niden (32) observed that sympathectomy reduced the pulmonary hypertension produced by experimental pulmonary embolism, whereas Courtoy and Salonikides (10) and Daley and associates (11) were unable to demonstrate this.

A number of investigations have shown that reserpine depletes from the organism not only 5-hydroxytryptamine but also noradrenaline, the principal mediator of the sympathetic nervous system (2, 7, 8, 15, 35). This depletion occurs both in the central nervous system (21) and in the peripheral nerves (31). There is also a loss of noradrenaline from the blood vessel walls, after which the vessels do not react as usual to sympathomimetic stimulation (4, 5, 9). It also has been demonstrated that the transmission of nerve impulses in the upper sympathetic cervical ganglion of the cat is inhibited in relation to the loss of noradrenaline caused by reserpine (30). It may thus be said that reserpine produces a «chemical sympathectomy».

In the present work the effect of reserpine in experimental pulmonary embolism was studied by means of the mortality rate, ECG findings, and histological features in the lungs.

METHODS

Male rats of Sprague-Dawley strain were given 4 mg/kg body weight of reserpine (Serpacil, Ciba) intraperitoneally on two consecutive days. Embolisation was performed three days after the last injection of reserpine. The embolus material used was *Lycopodium* spores (23.7×28.7 micra) suspended in physiological saline to make a 7 per cent suspension and injected into the jugular vein of rats under aether anaesthesia. The dosage of *Lycopodium* was about 10 mg/kg of body weight. Special care was taken to keep the rate of injection constant at 0.5 cc/min. A No. 14 needle was used in all cases. Electrocardiograms were taken with needle electrodes. In addition to the usual extremity leads unipolar chest leads were also taken from five points on the thorax, as done previously by Vartio and Halonen (43). (See Fig. 1.) Electrocardiograms were taken before and immediately after pulmonary embolism, and, in the case of survivors, one hour and two hours after embolism and on the following day. The success of the embolisation was controlled by

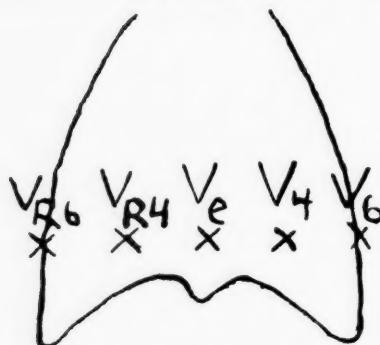


Fig. 1.

histological examination of the lungs of all rats. Rats that had not died as a result of the pulmonary embolism were killed on the following day. The lungs were fixed in 10 per cent formalin, sliced with an ice microtome, and examined without staining.

The effect of reserpine on possible vasocostriction was studied by the method of Sternberg and Tamari (40). They, and later Byrne (6), have demonstrated that in an anaesthetised animal the embolus particles enter the pulmonary capillaries, but in an unanaesthetised animal they remain in the arterioles, thus reflecting an active vasoconstriction. According to this method, 1 cc/200 g body weight of dialysed, formalinfree India ink was injected into the jugular vein of rats under aether anaesthesia, to unanaesthetised rats and to unanaesthetised rats which had been given 5 mg/kg of reserpine three hours previously. The animals were killed 5 min. after the inducement of embolism with an air embolus, the lungs were fixed in 10 per cent formalin, slices were made with an ice microtome, and the unstained specimens were examined. In the histological examination attention was paid to the amount of India ink particles in the capillaries. The arbitrary units +, ++, +++, ++++ were used to designate the amount of these particles per low power field. When making the histological examination it was not known from which group of rats the specimen was taken.

RESULTS

When pulmonary embolism was induced with *Lycopodium* spores the rats that did not survive the embolism usually died quickly. The mean survival times were 4 min. for the control rats and 2 min. for rats given reserpine. In the control group, 2 out

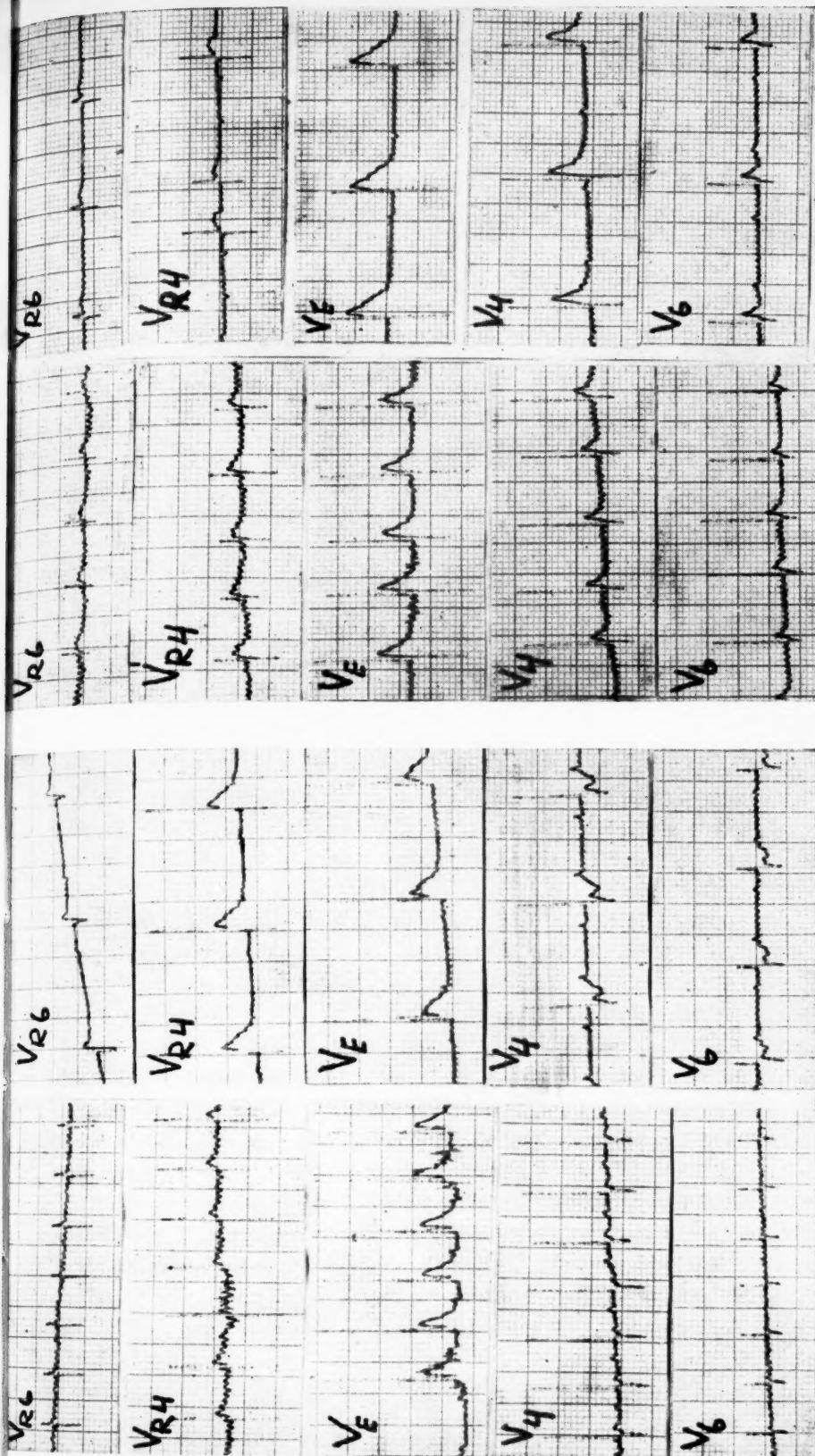


Fig. 2. — Some typical electrocardiographic tracings. a) Control rat. Left column: before pulmonary embolism. Right column: immediately after pulmonary embolism. b) Reserpine treated rat. Left column: before pulmonary embolism. Right column: immediately after pulmonary embolism. S-T elevation in V₄ and V₆, S-T depression in V₂₆ and V_F. High T in V₄. Postembolic bradycardia is seen in both tracings.

of 20 rats survived the embolism, while in the reserpine treated group 8 of 20 rats survived. The mortality in the latter case thus decreased to 60 per cent from the control group mortality of 90 per cent. The histological examination revealed *Lycopodium* spores in the lungs of all the rats.

The pulse rate was determined from the electrocardiogram. Before embolism it was 330/min. in controls and 310/min. in reserpine treated rats. Immediately after embolism the pulse rate of the controls was 220/min. and that of reserpine treated rats 230/min. In the two surviving control rats the pulse rate did not decline, and in six of the eight reserpinised rats that survived the pulmonary embolism there was a rise in the pulse rate after embolism.

The most common postembolic findings in the extremity leads were deepened S_1 , $S-T_1$ depression and $S-T_3$ elevation. In the control group there was S_1 deepening in 13 cases, $S-T_1$ depression in 8 cases and $S-T_3$ elevation in 4 cases. Two rats showed the S_1-Q_3 pattern. The rats given reserpine showed S_1 deepening in 7 cases, $S-T_1$ depression in 10 cases, and $S-T_3$ elevation in 2 cases. Chest leads showed changes in the $S-T$ segment only. This was elevated in the right leads in 19 control rats and depressed in the left leads in 9 control rats. The changes in the ECG of reserpine treated rats were less marked. There was $S-T$ elevation in the right leads in only 10 rats and $S-T$ depression in the left leads in 7 rats. Additionally, 7 reserpine treated rats had either T or $S-T$ elevation also in the left leads. This change was seen in only three of the control rats.

Two hours after the embolism there still were changes in the ECG of surviving rats, but on the following day the tracings were normalised. Fig. 2 shows two electrocardiograms of chest leads exhibiting typical changes.

On the injection of India ink into the jugular vein a marked blackness was observed in the limbs and oral mucosa of the animals. Examination of the amount of India ink particles in the capillaries revealed no difference in the findings in the lungs of unanaesthetised and unanaesthetised reserpine treated rats. In both groups the capillaries contained very few particles, estimated by the arbitrary unit of +. The lungs of anaesthetised rats contained large amounts of India ink particles (+++, ++++). Some typical histological photomicrographs are shown in Fig. 3.

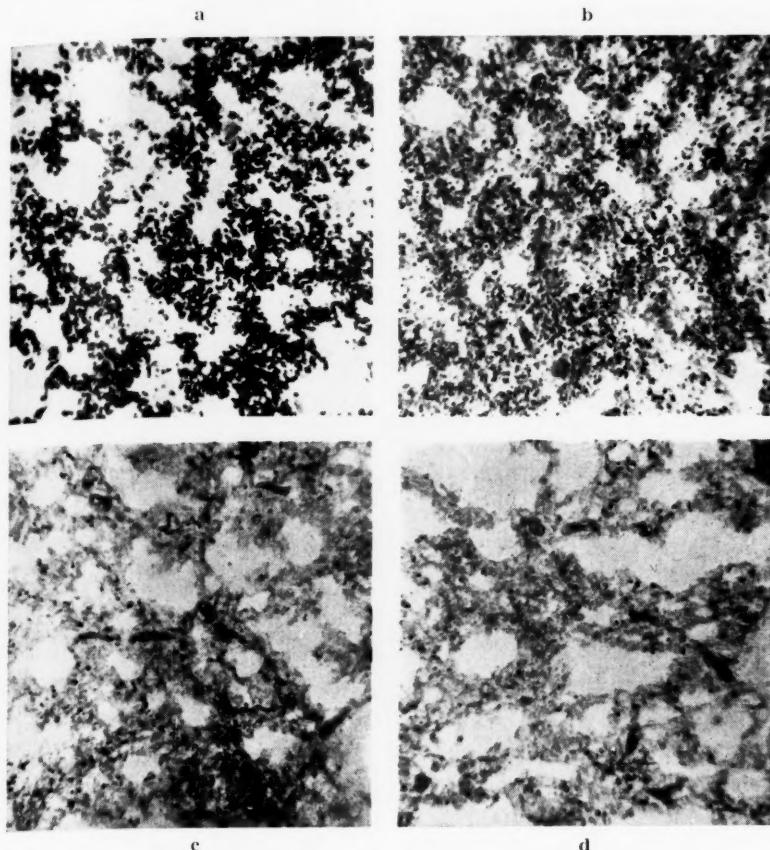


Fig. 3. — Histological pictures of the lungs injected with India ink.

a) and b) rat under aether anaesthesia, c) unanaesthetised rat and d) rat treated with reserpine and unanaesthetised. The amount of particles in capillaries measured with arbitrary units in a) ++++ and in b) ++. in c) + and d) +. Obviously reserpine did not alter the histological picture.

DISCUSSION

It was observed in the present work that reserpine slightly reduced the mortality due to pulmonary embolism. This observation accords well with the reports in which block of the ganglion stellatum is stated to be an effective means in the treatment of patients with pulmonary embolism. It also has been observed that cervical sympathectomy in rabbits increased to fourfold the lethal dose of pulmonary embolus material (44). However, pro-

cedures weakening or inhibiting the activity of the cervical sympathetic nerve are local measures, compared with the general depression of sympathetic activity of the organism produced by reserpine. For this reason, reserpine has a multiple mechanism of action, and in examining its effects one must consider both the central and the peripheral effects. It has been observed that the 5-hydroxytryptamine and noradrenaline contents of the brain are high in the diencephalon and mesencephalon, and especially in the vegetative centres in these regions (1, 46). It is in these regions that reserpine causes a marked depletion (21, 36).

In studying the effect of reserpine on vasoconstriction due to pulmonary embolism by the method of Sternberg and Tamari, it was not found to inhibit vasoconstriction. Thus the central effects of reserpine might serve as an explanation to the reduced mortality in the reserpine treated animals.

The effect of reserpine on the electrocardiogram of human subjects was studied, among others, by Harris (18), Schumann (39) and Székely and associates (41). According to them it produces bradycardia and elevation of the S-T segment and T. All these changes may be regarded as a result of increased vagotonus. In this work it was not possible to observe elevation of the S-T segment and T nor definite bradycardia. This result may have been due to the dosage of reserpine used and possible also to the increasing effect of aether anaesthesia on the pulse rate.

Although the ECG finding in pulmonary embolism in human subjects has been very accurately verified (14, 23, 24, 28, 29, 37), the electrocardiographic changes in experimental pulmonary embolism have been greatly variable and generally small. The most common changes in the extremity leads have been S_1 and Q_3 deepening, S-T₁ depression and S-T₃ elevation. Contrary to the above, Hochrein and Scheyer (20) observed in dogs S-T₂ and S-T₃ depression. In chest leads Vartio and Halonen (43) found S-T elevation in one or more leads. Flasher and associates (17) reported S-T depression in left chest leads and elevation in the right chest leads. The electrocardiographic changes in the present paper in the extremity leads of the control rats were similar to those most commonly observed earlier: S-T₁ depression, S-T₃ elevation and S_1 and Q_3 deepening. The changes in the chest leads of the control rats were in agreement with the observations of

Flasher and associates and consisted of S-T elevation in right leads and depression in left leads. These changes, according to several investigators, may be explained on the base of acute right ventricular strain. The reserpine treated rats exhibited the same changes; thus reserpine did not produce noteworthy change in the electrocardiographic status. It is true, however, that the electrocardiographic changes due to pulmonary embolism were present in a smaller number of rats in the reserpine treated group than in the control group, and, furthermore, seven rats in the reserpine treated group showed S-T or T elevation also in the left chest leads. The same phenomenon, however, was also seen in three control rats, and therefore it cannot be attributed to reserpine.

SUMMARY

The author studied the effect of reserpine pre-treatment in experimental pulmonary embolism in rats. The embolus material was *Lycopodium* spores. Reserpine was found to slightly reduce the mortality from pulmonary embolism. Pulmonary embolism was found to produce the following electrocardiographic changes: In extremity leads S₁ and Q₃ deepening, S-T₁ depression and S-T₃ elevation; in chest leads S-T elevation in right leads and depression in left leads. Reserpine did not alter the electrocardiographic features of pulmonary embolism. In the histological examination by the method of Sternberg and Tamari the administration of reserpine was found to have had no effect on the pulmonary vasoconstriction produced by pulmonary embolism. The reduced mortality was possibly a result of the depressing effect of reserpine on central sympathetic activity.

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EFFECT OF ACEPROMAZINE ON LIVER PARENCHYME IN THE WHITE RAT

by

MARTTI HORMIA and ARMO HORMIA

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During the past six years chlorpromazine has proved itself to be one of the most potent psychopharmacologic drugs as yet available. It, however, has side effects and also different kind of complications occur during the treatment. Therefore, attempts have been done to construct drugs which should have the beneficial effects of chlorpromazine without its side effects. Acepromazine, which contains an acetyl group instead of chlor in the structural formula, is one of these new therapeutic agents (4, 7).

In an earlier paper (5) it was shown that chlorpromazine in prolonged use causes considerable changes of the liver parenchyme in the white rat. Fat infiltration was the dominating feature in the histologic picture of this chlorpromazine influence. Besides this, necrosis and other regressive changes could be seen. The purpose of the present study was to throw light on the possible toxicity of acepromazine by studying its effect on the liver parenchyme. In order to compare the results with those obtained with chlorpromazine, similar methods were used as in the corresponding experiment with chlorpromazine.

MATERIAL AND METHODS

The effect of acepromazine on the liver parenchyme was studied by two groups of 10 white male rats. An accessory group of 10 rats served as controls. At the beginning of the experiment the

animals were 100 days of age. The drug was given subcutaneously once a day. Progressively increasing doses were used. The pharmacological effectivity of acepromazine has been shown to be the same or a little higher than that of chlorpromazine (1, 2, 3, 8). Therefore, acepromazine was given to the group I in the same dosage than had been used in the earlier experiment with chlorpromazine. On the first day 5 mg/kg body weight acepromazine ('Plegicil' Pharmacia) was given and the dose was increased by this same amount daily. The medication was continued during 25 days, after this the animals were killed. In the group II rapidly increasing doses of acepromazine were used. On the first day the dose was 20 mg/kg body weight and the dose was increased by this same amount daily. The medication was continued during 12 days, after this the animals were killed.

Each rat was weighed before the medication was started and after death, also the liver was weighed then. A specimen of the liver for microscopic study was taken immediately after death. Morphologic study took place after fixing in formol and staining with the van Gieson method. For glycogen sections were stained also with the Best's carmine method. The possible occurrence of fat was studied by cutting 10 μ frozen sections and by staining them with the Sudan III method.

RESULTS

In group I, receiving slowly increasing doses of acepromazine, the body weight had decreased by 4.4% during the treatment. In group II, receiving rapidly increasing doses the decrease was 5.5%. In the control group the weight of the liver was 3.8% of the total body weight. At the moment of killing the weight of the liver was in the group I as well as in group II 4.5% of the body weight and 4.3% of the body weight obtained before treatment. In both experimental groups the weight of the liver thus had increased to an equal extent.

By studying the liver microscopically it was seen that in group I receiving slowly increasing doses of acepromazine the liver parenchyme was considerably damaged. In the cells the cytoplasm had disintegrated, it was highly vacuolated and granular in appearance and the cell outlines were indistinct. In many cases some pyknosis

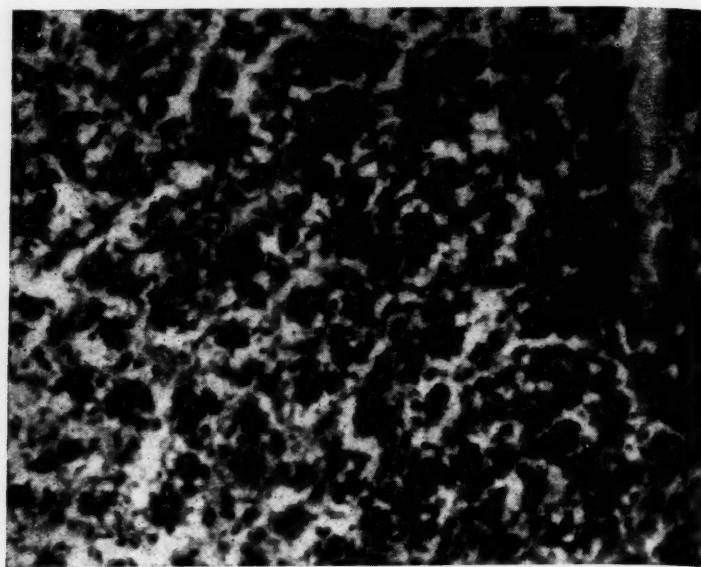


Fig. 1. — Photomicrograph of the rat liver showing granulation and vacuolation of the cytoplasm caused by prolonged acepromazine medication. Van Gieson stain, $\times 700$.

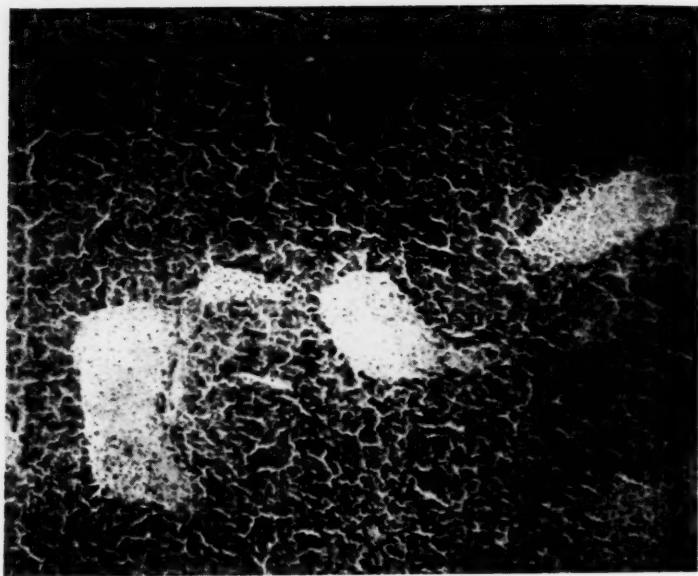


Fig. 2. — Photomicrograph of the rat liver showing necrotic areas caused by prolonged acepromazine medication. Van Gieson stain, $\times 100$.

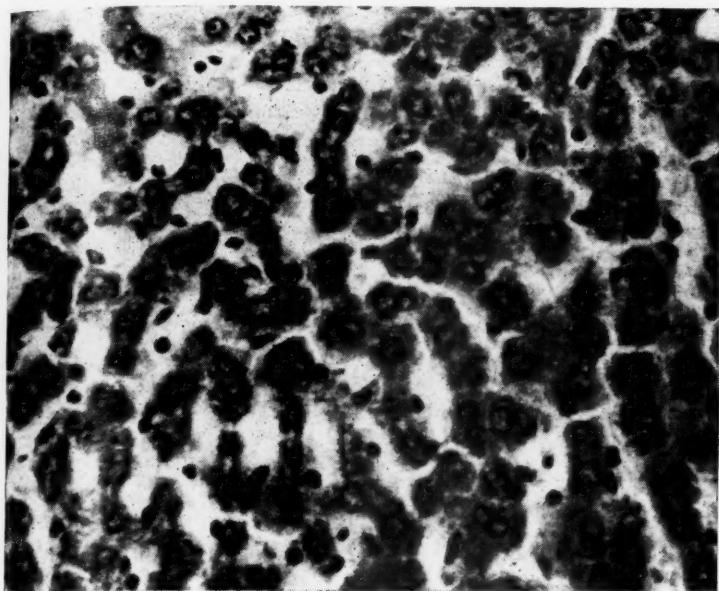


Fig. 3. — Photomicrograph showing liver parenchyme in the control group.
Van Gieson stain, $\times 700$.

of the nuclei was seen and in some cases small necrotic regions in addition. No intracellular fat could be demonstrated with Sudan III. In all cases the glycogen content of the cells was normal except in the necrotic regions, in which no glycogen was seen.

In group II receiving rapidly increasing doses of acepromazine the disintegration, vacuolated and granular appearance of the cytoplasm and pyknosis of the nuclei was present to the same extent as in the preceding group. Differing from the preceding group no necrosis could be seen. On the other hand, in the sections stained with Sudan III an intracellular accumulation of small Sudan positive fat droplets was seen at the periphery of the lobules. The glycogen content of the tissue was normal.

DISCUSSION

The results exposed above show that prolonged acepromazine medication in large doses causes parenchyme lesions of the liver in the white rat, namely disintegration, granular and vacuolated

appearance of the cytoplasm, pyknosis of the nuclei and necrosis of the parenchyme. These lesions were of almost the same degree in the group receiving slowly increased doses during a longer time and in the group receiving rapidly increased doses during a shorter time when the total quantity of the drug was nearby of the same level. No necrosis, however, was seen in the latter group. On the other hand, signs of a slight fatty degeneration could be seen in this group.

The same type of changes as described above were also seen in the former chlorpromazine experiment cited above. Also the functional hypertrophy of the liver was present in both series. Both acepromazine and chlorpromazine thus have toxic effects on the liver parenchyme but the essential difference in the acepromazine influence as compared to the chlorpromazine-caused parenchyme lesions of the liver is the milder degree of fatty degeneration. A pronounced fat infiltration in turn was the prominent feature of the chlorpromazine influence and was put in connection with an insufficiency in the function of the lipotropic factors (5, 6).

SUMMARY

The effect of acepromazine on liver parenchyme in the white rat was studied. A prolonged medication with large doses of acepromazine caused a considerable damage of the liver parenchyme. Its microscopical signs were disintegration, granular and vacuolated appearance of the cytoplasm, pyknosis of the nuclei and necrosis of the parenchyme. The vacuolated appearance of the cytoplasm was partly due to an intracellular accumulation of small fat droplets, partly to a vacuolar degeneration. These changes were of same type as those seen in connection with prolonged chlorpromazine medication described in an earlier paper. In clear contrast, acepromazine caused only a mild degree of fatty degeneration of the liver parenchyme. A pronounced fat infiltration, on the contrary, was the dominating feature of the chlorpromazine influence.

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UROPEPSIN EXCRETION DURING ONE AND SEVERAL DAYS

by

S. J. VIIKARI and KALEVI KOSKI

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Uropepsin first received attention during last century when Brücke studied it in 1861. The proteolytic enzyme, uropepsin, is in all probability identical with gastric pepsinogen. It enters the blood from the gastric mucous membrane and is then excreted into the urine by way of the kidneys. The mechanism of the excretion of uropepsin has been studied by Gregor and Schück (10). According to them there was a direct connection between uropepsin excretion and the endogenous creatine clearance. This points to the fact that uropepsin is excreted into the urine through glomerular filtration. The 24-hour-excretion of uropepsin corresponds closely with gastric digestion. The normal uropepsin excretion is 20—40 uropepsin units/hour. In Vartio's material (17) the average normal value was 46.2 ± 3.86 units/hour.

Since there are to be observed considerable variations in the excretion of uropepsin in different diseases as well in healthy subjects (1—3, 5—9, 11—20) the writers have considered it worth while to study uropepsin excretion in different persons during several days. In the study comparisons have also been made between the original values and those representing the average of the following determinations.

METHOD

Uropepsin determinations were made by the casein flocculation method described by West, Ellis and Scott. The time required for complete precipitation could thus be measured with an accuracy of 5 seconds.

RESULTS

The series consisted of 36 patients, of whom 12 were normal subjects, 11 duodenal ulcer and 13 gastric ulcer patients. Altogether 252 determinations were made on 24-hour urine samples, collected within 3 to 12 following days. The average values were 23.53 ± 1.77 units/hour for the normal cases, 63.93 ± 4.11 units/hour for the duodenal ulcer patients, and 53.72 ± 3.68 units/hour for the patients with gastric ulcer. When studying the average values of the first determinations and those obtained by all measurements no great differences are to be observed between the respective figures (Table 1).

TABLE 1
AVERAGE UROPEPSIN VALUES IN SEVERAL PATIENT GROUPS

	First Determination	3—12 Determinations
Normal subjects	25.29 ± 4.83	23.53 ± 1.77
Duodenal ulcer	68.07 ± 14.72	63.93 ± 4.11
Gastric ulcer	56.16 ± 8.16	53.72 ± 3.68

DISCUSSION

As the present material is comparatively small it is only to be expected that the figures obtained differ somewhat from the corresponding values *e.g.* in Vartio's series. This difference is particularly noticeable in the excretion values established for normal subjects. In Vartio's material average was 46.2 ± 3.88 units/hour; our respective figures being 25.29 ± 4.83 (first determinations) and 23.53 ± 1.77 . With respect to gastric ulcer patients the two values are of the same extent: Vartio 58.7 ± 6.1 units/hour; the present material 56.16 ± 8.16 (first determinations) and 53.72 ± 3.68 units/hour. Our figures for duodenal ulcer patients

are somewhat lower than those obtained by Vartio: Vartio 87.7 ± 15.41 ; the present material 68.07 ± 14.72 (first determinations) and 63.93 ± 4.11 . The fact that there is hardly any difference between the average values obtained at the first determinations and those obtained by several measurements, is of particular significance. Still, in individual cases the difference between the first determination and the average of the subsequent ones may be quite considerable. As an example we may mention one gastric ulcer patient, whose first determination gave 100 units/hour but the average value for 5 days reached only 31.4 units/hours. Similarly the original excretion level of one duodenal ulcer patient was 146.5, and that expressing the 8 days' average was 75.75 units/hour. The opposite is supplied by the healthy subject whose first level was 13.9 and the average for 9 days 22.5. units/hour.

SUMMARY

The uropepsin excretion of 36 persons was determined by the West-Ellis-Scott method. Of the subjects 12 were normal, 11 duodenal ulcer and 13 gastric ulcer patients. Average values were calculated on the first and on all the determinations. The averages for the normal cases were 25.29 ± 4.83 (first determination) and 23.53 ± 1.77 ; for duodenal ulcer patients 68.07 ± 14.72 (first determinations) and 63.93 ± 4.11 ; for gastric ulcer patients 56.16 ± 8.16 (first determinations) and 53.72 ± 3.68 units/hour. In the present material there was hardly any difference between the average values of the first determinations and those of the total determinations. In individual cases even considerable differences could be observed between the first 24-hour excretion and the average excretion for several days.

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FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF HELSINKI, AND THE
DEPARTMENT OF MEDICAL CHEMISTRY, UNIVERSITY OF TURKU

PRECIPITATION OF POLYSACCHARIDES AS BASIS OF HISTOCHEMICAL REACTIONS

by

ANTTI TELKKÄ and EINO KULONEN

(Received for publication April 3, 1959)

The purpose of this paper is to show that mucin precipitation reaction can be applied as a histochemical technique. There exists a wealth of knowledge on the precipitation of acid polysaccharides with proteins (8) and recently, with quaternary cetyl ammonium bases in the presence of varying salt and hydrogen ion concentrations (6, 7). This principle has been used also for the staining of paper electrophoretic strips (3).

Chondroitin sulfate and hyaluronate form precipitates with protein, if the pH is on the acid side of the isoelectric point of the protein. The amount of the precipitate depends on the degree of polymerization of the polysaccharide (2). The precipitate first increases and then decreases during the depolymerization (1, 3) and finally no precipitate is formed. The presence of excess salt inhibits the precipitation.

The doubt may be expressed that the carboxyl groups of the acid polysaccharides in the tissue may not be available to react with extra protein. From the synovial fluid the hyaluronic acid can be isolated as a protein complex, which does not precipitate by the addition of acetic acid but forms a mucin clot if serum albumin is simultaneously added (5). We have found a similar complex from umbilical cord extract.

METHODS

With «spot test» on Whatman n:o 1 paper it was shown that umbilical cord hyaluronate-protein complex could be stained with the staining solution presented below. When the color was estimated with the densitometer, the expected relationship to the amount was observed. When the depolymerized hyaluronate was used, the stain was decreased.

Preparation of the Staining Solution. — Tetrabromphenolsuphonphthalein (Merck) was added in excess into 1% solution of serum albumin until the fluid was very dark. The solution was dialyzed against running tap water for a week when it became bluish. An equal volume of 0.1 M/pH 4.7 acetate buffer was added. For the control staining a part of the solution was alkalized with conc. ammonia to about pH 8. The washing fluid contained the acetate buffer only, and the hydrogen ion concentration was adjusted as in the staining solutions.

Procedure. — The tissues were fixed for 24 hours in 96 per cent ethyl alcohol. The specimens were dehydrated, embedded in paraffin in the usual manner and sectioned at 10 μ . After deparaffinization, the mounted sections were passed through a series of alcohols (absolute, 96 per cent, 70 per cent and 50 per cent) to the staining solution for 24 hours. The control sections were treated with an identical solution with pH 8.0. The sections were washed with fluids at respective hydrogen ion concentrations, dehydrated in alcohol, cleared in xylene and mounted in Canada balsam. Also frozen sections were used with equal results.

RESULTS

Following tissues were used: rat eye, bone and cartilage, intestinal mucous membrane, synovial tissue and skin, human umbilical cord and fibrinoid. In the eye and umbilical cord the experiment was successful (Fig. 1). In the others the control sections stained so darkly that the possible difference could not be evaluated. Treatment with trypsin did not appreciably influence the results in ocular tissues.

DISCUSSION

The expected result was obtained in the eye and umbilical cord only. In the others the control sections also stained intensively. Two explanations are offered: (a) the acidity of the polysaccharides (with high content of esterified sulfate) is so high that the precipitate may be formed especially as the salt strength is low; (b) the insoluble tissue proteins may adsorb extra protein with the

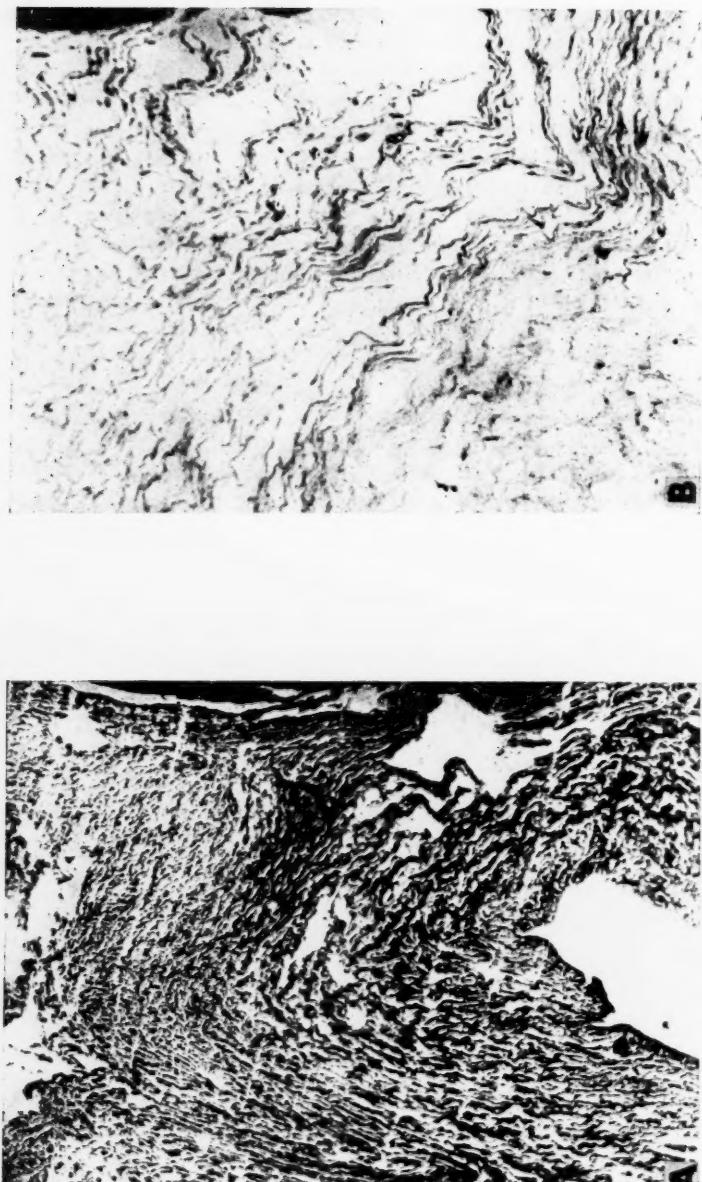


Fig. 1. — Human umbilical cord. A. Section stained in staining solution with pH 4.7. B. Control section stained in the alkalinized staining solution, pH 8.0. ($\times 300$).

dye, quite independently of the pH or the salt concentration. In this case the sclera of the eye should also stain, but nevertheless this seems rather a reasonable explanation. Conditions are sought, were the adsorption could be minimized. We know from the preliminary experiments that the salt concentration is critical.

ACKNOWLEDGEMENTS

The financial support from the Sigrid Jusélius Foundation and the Rheumatism Foundation is gratefully acknowledged.

SUMMARY

Connective tissue polysaccharides have been precipitated *in situ* as mucins with serum albumin. The bound protein was stained with tetrabromphenolsulphonphthalein. The attempts were successful with ocular tissues and umbilical cord but several tissues stained (presumably by adsorption) also in conditions where mucin precipitate is not formed.

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FROM THE DEPARTMENT OF PHYSIOLOGY, AND THAT OF NEUROLOGY AND
PSYCHIATRY, UNIVERSITY OF TURKU

EFFECT OF PSYCHIC TENSION ON CAPILLARY RESISTANCE

by

PANU HAKOLA, RAUNO HEIKINHEIMO and ANTTI KAUPPILA

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Da Silva Mello in 1929 was the first to call attention to the fact that measurements of capillary resistance (CR) gave above normal values if the subject was under tension (1). After him this observation has been made by several investigators, who have also tried to eliminate the effect of disturbing psychic factors by different means; *e.g.* they have made the patient sit for a while in the room where the measurements are being made thus letting him see the test carried out on another subject; or readings obtained during the first few minutes have not been taken into consideration. There has been made, however, no detailed study on the effect of psychic factors on capillary resistance.

THE OBJECT, MATERIAL AND METHODS OF THE PRESENT INVESTIGATION

The object of the present work was to investigate whether emotional tension has an effect and, if any, of what kind, on the CR of test subjects.

»Psychic tension» is a fairly comprehensive conception. Emotionally it can be experienced in many different forms, *e.g.* as restlessness, fear, or activity, and it may be due to a number of various causative factors, *e.g.* danger, expectation, a difficult

task, etc. By psychic tension the authors understand here the emotional tension that was caused by the test situation without any attempt at its specification with respect to its character.

The series consists of 47 medical students and 10 inmates of the mental hospital. Their CR's were measured during, and immediately after, the presence of psychic tension as well as on the following day most exactly 24 hours after the first measurement. In the medical students the psychic tension was brought about by the fact that they were going to have an examination of great significance; the measurements were taken from one hour and a half to 15 minutes before it. In the inmates the tension was due to their fear of having to undergo an electric shock immediately after the first measurement. No shock was given, however.

The measurements of CR were made in the supraclavicular region by means of a selfconstructed modification (2) of v. Borbely's apparatus (3). The radial pulse of every subject was also taken in connection with the CR measurement. Each medical student was also inquired as to his own conception of the tension before the examination.

By CR we mean the lowest depression capable of producing, within one minute, two, or more, petechiae in the center of the suction cup with a 2 cm diameter. According to previous studies (2), a deviation in CR which exceeds 1 cm g is to be considered significant when CR is less than 10 cmHg and, for higher CR values, if it exceeds 2 cmHg.

RESULTS

When comparing the values obtained immediately ($= \frac{1}{2}$ hour—2 hours) after the relaxation of the tension with those measured on the following day, it was observed that only in few individual cases there were significant differences between the two readings and that in the material as a whole there was nothing to indicate a higher level for either value. A comparison of these two values with those obtained during the tension showed, that there was, as a rule, a much greater regularity between the tension values and the readings taken 24 hours after it, which, no doubt, had been affected less by disturbing factors than those obtained immediately after the relaxation. Consequently the following comparisons have been

TABLE 1

THE DISTRIBUTION OF THE MATERIAL IN THE DIFFERENT GROUPS WITH RESPECT
TO THE CHANGE IN CR DUE TO PSYCHIC TENSION

Change in CR cmHg	+ = CR increased, — = CR decreased		
	Medical Students	Inmates	Total
+ 13	0	1	1
+ 12	0	0	0
+ 11	0	0	0
+ 10	0	0	0
+ 9	0	0	0
+ 8	0	1	1
+ 7	1	0	1
+ 6	3	0	3
+ 5	2	0	2
+ 4	3	2	5
+ 3	12	2	14
+ 2	11	1	12
+ 1	2	1	3
0	2	2	4
- 1	5	0	5
- 2	5	0	5
- 3	1	0	1
Average	+ 1.9	+ 3.8	+ 2.23

made between the tension values and those obtained 24 hours after the relaxation of the tension.

Table 1 shows the difference (increase or decrease) between the CR of each subject measured during the tension and the one obtained on the following day. The table shows that CR was definitely higher (level of significance less than 0.1%) during the tension than when the tension did not exist. The average increase of CR during the tension was 2.23 cmHg (with standard error ± 0.38 cmHg). There were some differences between the student group and the inmate group.

In Table 2 the tension values have been compared with the relaxation CR values. No statistically significant difference is observed between the CR's of various original levels though it appears as if the absolute change in lower original values were slightly greater than that in the originally higher values.

TABLE 2
CHANGES IN DIFFERENT CR LEVELS DUE TO PSYCHIC TENSION

CR at Rest cmHg	Change in CR in cmHg											Total	
	-3	-2	-1	0	+1	+2	+3	+4	+5	+6	+7		
0—5	—	—	—	—	2	3	—	—	—	—	—	5	
6—10	1	—	2	2	1	8	9	2	—	—	1	1	27
11—15	—	1	—	1	1	2	2	1	—	3	—	1	12
16—20	—	2	1	1	—	—	—	2	2	—	—	—	8
21—25	—	1	2	—	—	—	—	—	—	—	—	—	3
26—30	—	1	—	—	—	—	—	—	—	—	—	—	1
31—35	—	—	—	—	1	—	—	—	—	—	—	—	1
Total	1	5	5	4	3	12	14	5	2	3	1	1	57

Table 3 shows the relation between the change in CR and the radial pulse rate taken during the psychic tension. In table reveals highly significantly (level of significance 0.1%) that the increase in CR was highest in the persons that had the highest pulse rate. When comparing the change in CR with that seen in the pulse during the tension or with the pulse rate obtained during rest there is not to be observed such a high correlation, which would indicate that two factors were both responsible for the above correlation.

To compare the change in CR with the amount of tension as experienced by each subject according to his own information,

TABLE 3
THE CHANGE IN CR DUE TO PSYCHIC TENSION COMPARED WITH THE SUBJECT'S SIMULTANEOUS RADIAL PULSE RATE

Pulse times/m.	Change in CR in cmHg											Total	
	-3	-2	-1	0	+1	+2	+3	+4	+5	+6	+7		
70—79	1	1	3	—	—	—	4	—	—	1	—	—	10
80—89	—	1	1	3	—	5	1	1	1	—	—	—	13
90—99	—	3	1	1	2	4	3	—	1	—	1	—	16
100—109	—	—	—	—	1	2	3	2	—	—	—	1	9
110—119	—	—	—	—	—	—	2	2	—	1	—	—	5
120—129	—	—	—	—	—	—	1	—	1	—	—	1	3
130—139	—	—	—	—	—	1	—	—	—	—	—	—	1
Total	1	5	5	4	3	12	13	6	2	3	1	1	57

the medical students were asked whether they considered to be »slightly», »moderately», or »very» excited. In nineteen that regarded themselves as »slightly» excited the average rise in CR was 2.6 cm Hg. The corresponding figure for 21 »moderately» excited students was 0.9 cm Hg, and that for the 7 »very» excited ones, 2.7 cm Hg.

DISCUSSION

It is evident on the basis of the present series that a rise in CR took place because of psychic tension. The fact that the greatest increase in CR occurred in those who also had the greatest pulse rate indicates a correlation between the extent of the rise in CR and the intensiveness of the psychic tension.

It was also observed that in subjects with a lower CR level the increase was slightly larger than in subjects with a higher CR level. An explanation may either be found in the fact that subjects with lower CR were more neurolabile and consequently more susceptible to psychic tension or that a low CR is, in general, more sensitive to stimuli than a higher one.

According to the information submitted by the subjects themselves, such as exhibited the greatest change in the CR were, on one hand, the least excited and, on the other, the most excited ones, whereas those with only a slight variation in the CR were in the group labelled »moderately excited». Since it is well-known that the subjective estimation of one's psychic state is extremely difficult and may even yield quite misleading results no conclusions can be drawn on the basis of this finding. It is possible, however, that those who appeared to be the least excited were actually very excited but were not conscious of their tension or concealed it.

When considering the observed rise in CR in the light of the other changes in CR that are known to be due to the influence of the general nervous system, it is seen that, in addition to psychic tension, electric shock is the only one observed to produce an increase in CR (4) whereas there has been noticed a decrease in it when the general nervous system is stimulated mechanically or by means of x-rays as well as after a concussion of the brain (5; cf. bibliographies of Frohn and Heikinheimo & Sallas).

It is possible that the influence of psychic tension on CR acts through the increasing effect of the added adrenaline content of the

tissues on the CR (6). This is supported by the fact that CR, increased by psychic tension, may fall almost momentaneously, which is observed on the occasion when the tension is due to the patient's fear of the CR measurement and disappears as soon as the measurement begins and proves to be completely painless and harmless.

SUMMARY

The capillary resistance (CR) of 47 medical students and 10 inmates of a mental hospital was measured during and after psychic tension due to the test situation. The measurements were made in the supraclavicular region, utilizing the suction method.

CR was observed to be higher during the tension than after its relaxation. The increase in CR was slightly greater in those who exhibited the lowest CR level when not excited. The increase was also found to be greatest in those with the quickest pulse during the tension.

The subjective estimation given by the students with respect to their tenseness did not correlate with the range of the changes observed in CR.

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CHANGES IN THE MICROBIAL AMINO ACID METABOLISM INDUCED BY 4-DEOXYPYRIDOXINE

by

J. JÄNNES and T. LAHIKAINEN

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The antagonist of pyridoxine, 4-deoxypyridoxine, was already in the 1940's known as an inhibitor of transamination reactions [Umbreit and Gunsalus (9), Umbreit and Waddel (10)]. In 1953 Rabinowitz and Snell (7) observed that the addition of deoxypyridoxine to media containing ammonium salts and asparagine as sources of nitrogen could not in any way affect the growth of a mutant strain of *E.coli*. They used a medium described by Lederberg and Tatum (4), and the doses of 4-deoxypyridoxine were only 1 mg per 1 ml of medium.

The purpose of the present study was to clarify if the presence of 4-deoxypyridoxine in a medium containing DL-asparagine as the only source of nitrogen could in any way affect the final amino acid composition of the medium when normal strains of *E.coli* were used as test organisms. In the tests described below the incubation time was considerably long, 7 to 8 days.

The medium used by the writers was composed of the following ingredients:

Glucose	100 mg
DL-Asparagine	50 mg
Na ₂ HPO ₄ 2 H ₂ O	10 mg
NaCl	2 mg
Na ₂ SO ₄	1 mg
Tap water to make 10 ml.	

The p_H was adjusted to 7.0 with Na₃PO₄

4-deoxypyridoxine was kindly supplied by Hoffman-La Roche, Basle.

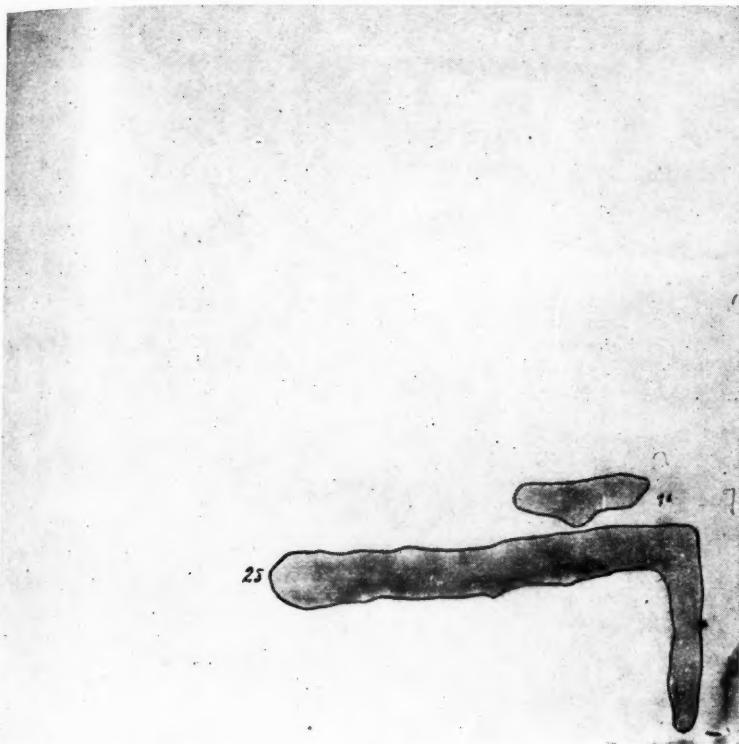


Fig. 1. — Before inoculation.

Asparagine was chosen because of the good adaptability of most micro-organisms to this amide.

To test tubes containing 10 ml of this medium, 4-deoxy-pyridoxine was added in increasing amounts, *e.g.*, 10, 20, 30, 40 mg per test tube. After final adjustment to neutrality with Na_3PO_4 the tubes were autoclaved and inoculated with 2 drops of a fresh culture of *E.coli*.

After 7 days incubation at 37°C the tubes were centrifuged and the clear supernatants were treated with Amberlite IR 120 resins in acid form and eluted with 1 N ammonium hydroxide. Thereafter the samples were evaporated *in vacuo* and made up to 1 ml. Exactly the same amount of each sample was then used for chromatographic analysis. The method described by Parry (6).



Fig. 2. — After normal growth of Strain No. 1.

was followed with some minor modifications. Two dimensional paper chromatograms were prepared by using phenol : water and butanol : acetic acid : water (4 : 1 : 5) as solvents and the spots were developed with 0.25 per cent ninhydrin solution.

When the amount of 4-deoxypyridoxine exceeded 20 mg per test tube, the inhibitory effect of 4-deoxypyridoxine on the growth was distinct. The lag phase lasted about two days, and not until the third day was the growth of the bacteria noticeably accelerated.

Difficulties were encountered when trying to measure the turbidity values after the growth period because the bacteria could not be evenly distributed. However, during the growth period clear differences were visible. In all the tests containing 4-deoxypyridoxine the growth was less than in other tests.

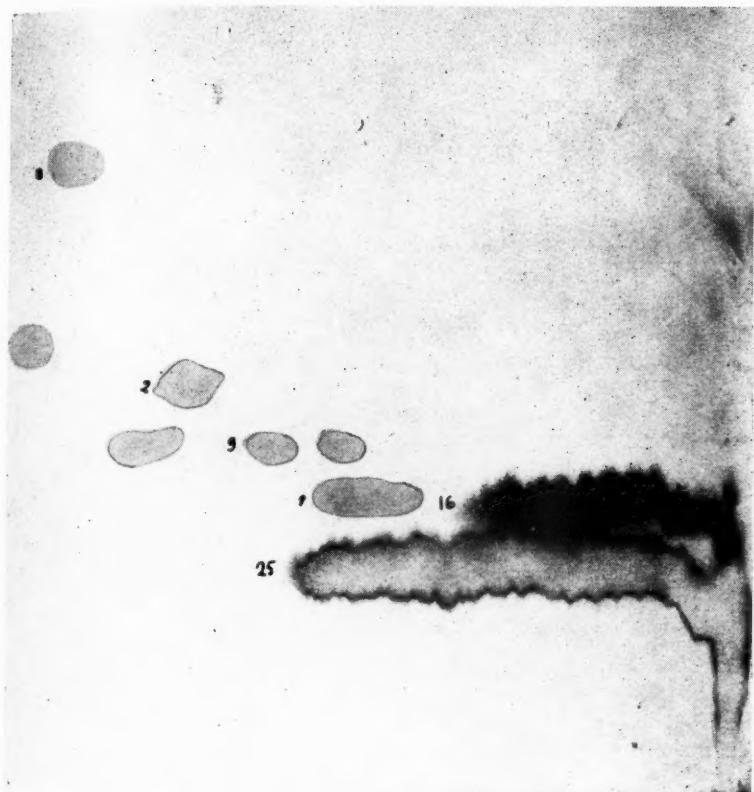


Fig. 3. — After growth in the presence of 40 mg deoxypyridoxine.
Strain No. 1.

Pyridoxine hydrochloride, pantoyl taurine and the sodium salt of DL-methyl pantothenic acid had no inhibitory effect on the growth, nor on the final amino acid composition of the media.

In the presence of 4-deoxypyridoxine in the culture media, differences in the chromatograms were noticeable.

Fig. 1 gives the result of the chromatographic analysis of the medium before inoculation. It can be seen that in addition to the spot of asparagine (25), also the spot of aspartic acid (16) is present.

Fig. 2 shows the chromatogram of the medium after the growth period in the absence of 4-deoxypyridoxine, and in Fig. 3 the effect of 40 mg of deoxypyridoxine in the culture media can be seen. The large spots of asparagine (25) and aspartic acid (16)

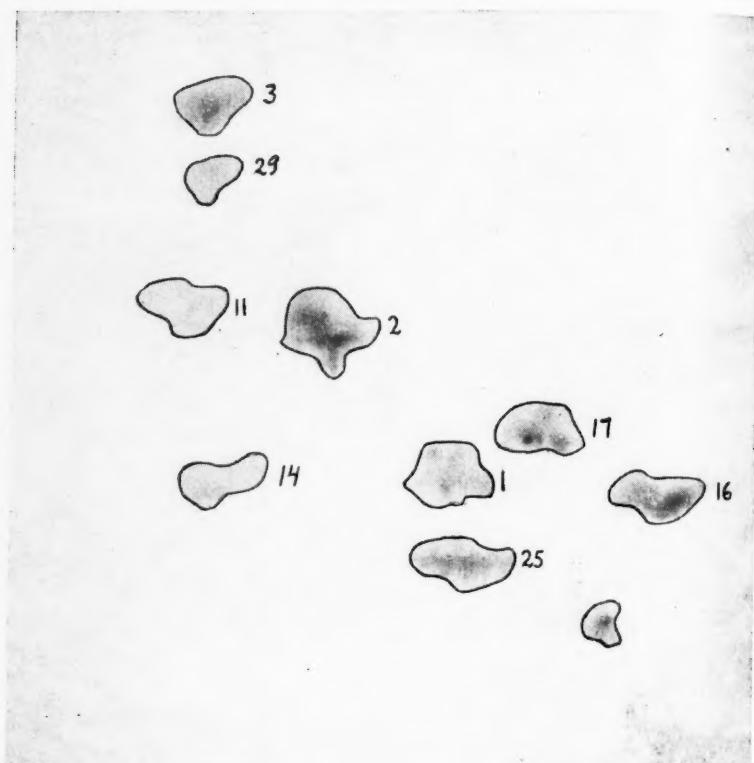


Fig. 4. — After normal growth of Strain No. 2.

are in the right lower corner of the papers. In Fig. 2 the spots of valine (3), alanine (2), glycine (1), threonine (9), and γ -amino butyric acid (29) can be seen. In Fig. 3 the following spots could be identified: asparagine (25), aspartic acid (16), valine (3), alanine (2), threonine (9), and glycine (1). In addition to these spots, three weak spots could be seen. When compared to Fig. 2, the most characteristic finding was the disappearance of the spot of γ -amino butyric acid.

Because the differences seen in these chromatograms were not clear enough, similar experiments were carried out with another strain of *E.coli*.

The chromatograms presented in Figures 4, 5, and 6 were obtained with strain No. 2. Before chromatographing, proline

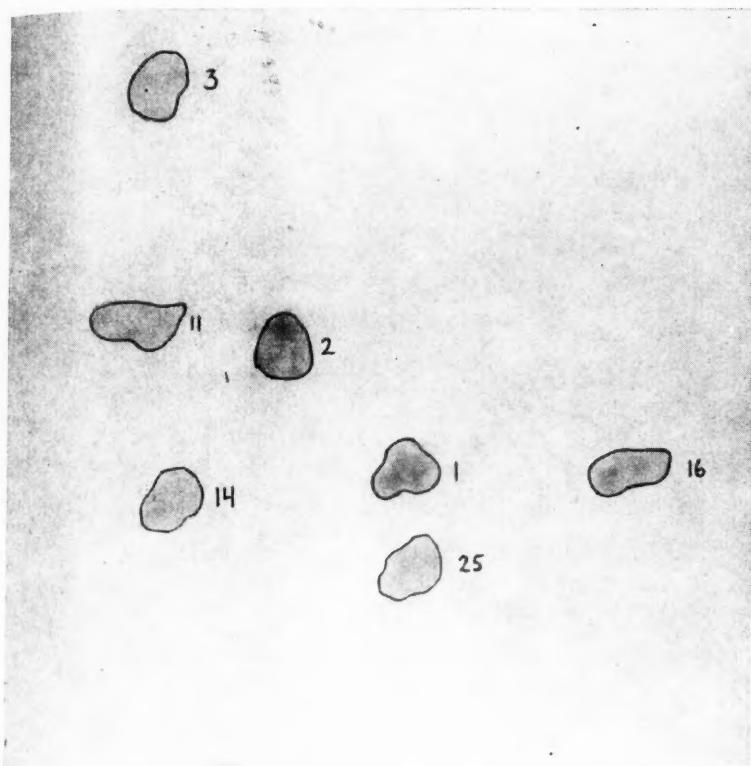


Fig. 5. — After growth in the presence of 20 mg of deoxypyridoxine.
Strain No. 2.

was added to help the identification of the spots. Fig. 4 shows the chromatogram of the media in the absence of 4-deoxypyridoxine. The spots of glycine (1), alanine (2), valine (3), a basic amino acid which is probably arginine (14), aspartic acid (16), glutamic acid (17), asparagine (25), γ -amino butyric acid (29) and proline (11) were obtained. In addition, there was a weak spot below asparagine which was not identified. Fig. 5 shows the chromatogram when 20 mg of deoxypyridoxine had been added into the medium and in Fig. 6 the effect of 30 mg of deoxypyridoxine can be seen. The most characteristic phenomena shown by these chromatograms is the complete disappearance of the spot of glutamic acid (17) and also that of γ -amino butyric acid (29).

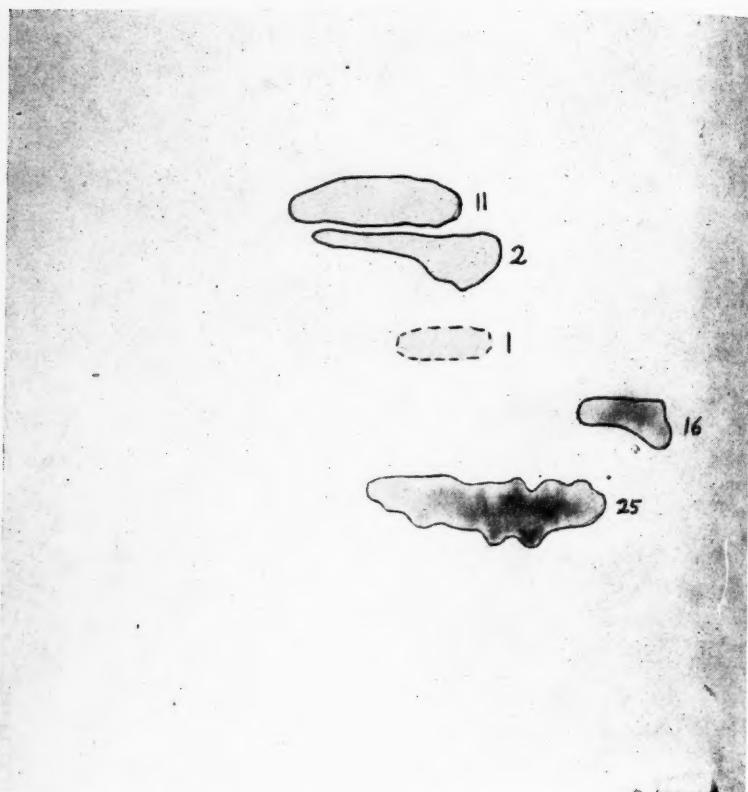


Fig. 6. — After growth in the presence of 30 mg of deoxypyridoxine.
Strain No. 2.

DISCUSSION

The only plausible explanation to the findings described in this paper is the inhibition of aspartic acid-glutamic acid transamination reaction by 4-deoxypyridoxine. As stated in 1937 by Braunstein and Kitzmann (2), the transamination reaction plays a very important part in the metabolism of aspartic acid. The presence of this system in bacterial cells was first demonstrated by Lichstein and Cohen in 1944 (5). This reaction depends upon the presence of pyridoxal phosphate [Snell (8)] and the addition of deoxypyridoxine inhibits this enzyme system, although it is not able completely to inhibit the growth of the bacteria. The disappearance of the spot of γ -amino butyric acid, seen in chromato-

grams No. 3, No. 5, and No. 6, is also due to the lack of pyridoxal phosphate. Pyridoxal phosphate is required for the decarboxylation of glutamic acid to give γ -amino butyric acid [Bellamy, Umbreit and Gunsalus (1), Gale (3)]. The production of γ -amino butyric acid by *E.coli* has been demonstrated by Woiwod and Proom (11).

According to the experiments presented here, the growth of the bacteria is possible even under such conditions where some of the most important enzyme systems are inhibited.

The changes in the amino acid metabolism can be followed in also this manner, since the disturbances in the metabolism are reflected in the environment.

SUMMARY

Normal strains of *E.coli* were grown in media containing 4-deoxypyridoxine in amounts of 10, 20, 30, and 40 mg per 10 ml of the culture media, asparagine being the only source of nitrogen.

Deoxypyridoxine in these concentrations retards the growth which results in a different amino acid pattern when the media is chromatographed after the growth period. The most striking difference is the disappearance of the spots of both glutamic acid and γ -amino butyric acid when deoxypyridoxine had been added.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY
OF HELSINKI

SUGAR SPECIFICITY OF PLANT HEMAGGLUTININS (LECTINS)

by

O. MÄKELÄ¹, PIRJO MÄKELÄ and RAIMO LEHTOVAARA

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Several plants contain proteins that agglutinate red blood cells, as do many antibodies (6, 7). For these proteins the name »lectin« has been suggested (3). Lectins can be as specific as animal antibodies in that they react only with erythrocytes of certain blood groups.

The ABO specific lectins — as well as the ABO specific antibodies — are inhibited by simple sugars, mainly components of blood group polysaccharides (9, 5, 6, 7). The explanation of this phenomenon is that the »receptors» of the lectins on the erythrocyte surface are carbohydrates, and that the sugars whose structure most closely resembles the specific (sugar) group of the red cell receptor attach themselves to the active site(s) of the lectin molecule thus blocking them.

There are also »unspecific» lectins which react with red cells of all human beings. Many of these lectins, too, are inhibited by simple sugars but these sugars are not necessarily components of blood group polysaccharides (6, 7).

There seem to be certain further correlations between the structure of sugars and their ability to neutralise different lectins. The present report deals with these rules.

¹ Aided by a grant from the University of Helsinki.

MATERIAL AND METHODS

Lectins. — The following species were chosen as representatives of different types of lectins: *Pisum sativum* L., *Cytisus sessilifolius* L., *Bandeiraea simplicifolia* Benth., *Crotalaria juncea* L., and *Lotus tetragonolobus* L.

Sugars. — The following sugars were used in all experiments: D-glucose, D-glucosamine, N-acetyl-D-glucosamine, D-glucuronic acid, D-gluconic acid, D-xylose, D-mannose, D-fructose, L-sorbose, D-galactose, D-galactosamine, N-acetyl-D-galactosamine, D-fucose, L-arabinose, D-talose, L-galactose, L-fucose, D-arabinose, D-ribose, D-digitoxose, L-rhamnose, D-allose, D-altrose, maltose, sucrose, turanose, trehalose, cellobiose, gentiobiose, melibiose, raffinose, lactose.

Erythrocytes. — *Cytisus sessilifolius* and *Lotus tetragonolobus* lectins are blood group O(H) specific and *Bandeiraea simplicifolia* lectin is B specific. *Pisum sativum* and *Crotalaria juncea* lectins are «nonspecific», they agglutinate erythrocytes of any blood group equally. The experiments with *Bandeiraea* lectin were carried out using human erythrocytes of blood group B and those with other lectins using O cells. The cells were washed three times with saline, in which a 3% suspension was then made.

Preparation of Lectin Solutions. — The seeds were ground in a mortar. One part of seed powder was mixed with nine parts of saline and the mixture was incubated at + 37° C for 2 hours. After centrifugation (3 000 r.p.m. for 20 minutes) the supernatant was used.

Preparation of Sugar Solutions. — 240 millimolar solutions were made in saline and neutralised before use if necessary.

Agglutination Inhibition Test. — The agglutination titres of the lectin solutions were determined and a dilution was chosen which contained 2 to 4 times the minimum amount of lectin needed for distinct agglutination. Of this dilution 0.05 ml amounts were distributed to round-bottomed tubes (10 × 100 mm). Of the 240 millimolar sugar solutions doubling serial dilutions were made in saline. Of these dilutions 0.05 ml was added to the tubes containing lectin. After 30 minutes incubation at + 20° C 0.05 ml of red cell suspension was added to each tube, and after further 2 hour's incubation at 20° the agglutination was read with the naked eye.

RESULTS

The results are in Tables 1 and 2. The figures give the final dilution of sugar. Of the sugars tested (see above) only those are included in the tables which inhibited some of the lectins at a final concentration of 80 millimoles per litre. None of the monosaccharides tested inhibited *Cytisus sessilifolius* lectin, and it is not included in Table 1.

DISCUSSION

The Monosaccharide Specificity of Lectins. — The members of a homomorphous sugar series (sugars having the same configurations for the atoms that compose the pyranose ring) show marked chemical and physical (11), and biological similarities. Thus it appears

TABLE 1
THE AGGLUTINATION INHIBITION EFFECT OF MONOSACCHARIDES

	Minimum Amount of Sugar (Millimoles per Litre) Inhibiting the Action ¹ of			
	<i>Pisum sativum</i> Lectin	<i>Bandeiraea simplicifolia</i> Lectin	<i>Crotalaria juncea</i> Lectin	<i>Lotus tetragonolobus</i> Lectin
D-glucose	1.25	>80	>80	40
D-glucosamine	5	>80	>80	>80
N-acetyl-D-glucosamine	2.5	>80	>80	10
D-xylose	>80	>80	>80	>80
D-mannose	0.6	>80	>80	40
D-galactose	>80	0.6	5	>80
D-galactosamine	>80	5	>80	>80
N-acetyl-D-galactos- amine	>4	0.4		>4
D-fucose	>80	2.5	20	>80
L-arabinose	>80	5	20	>80
D-talose	>80	20		>80
L-galactose	>80	>80	>80	1.25
L-fucose	>80	>80	>80	0.2
D-arabinose	>80	>80	>80	5
D-digitoxose	>80	>80	>80	5
D-ribose	>80	>80	>80	10
L-rhamnose	>80	40	>80	>80

¹ The tubes contained 2 to 4 agglutinating doses of lectin. For full explanations see the text (Discussion).

that enzymes which hydrolyse the hexoside members of each series also hydrolyse the glycosides of the other members of each series (10).

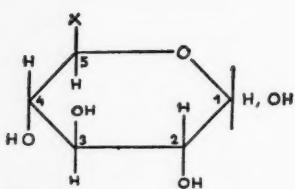
The members of a homomorphous series often show similar characteristics in agglutination inhibition tests, too. Thus D-galactose, D-fucose and L-arabinose (sugars of the »D-galactose type« of Fig. 1) inhibited the agglutination by *Bandeiraea* and *Crotalaria* lectins, and L-galactose, L-fucose and D-arabinose (sugars of the »L-galactose type« of Fig. 1) inhibited the agglutination by *Lotus* lectin (see Table 1).

Furthermore, many lectins are inhibited by members of two homomorphous series. These series differ with regard to the placement of -H and -OH groups in carbon atom 2. Thus *Pisum* lectin is inhibited not only by D-glucose but by D-mannose as well, *Bandeiraea* lectin is inhibited by D-galactose series and by D-talose (Table 1), and *Lotus* lectin by L-galactose series, D-ribose (Table 1) and by 6-deoxy-L-talose (9) (see Fig. 1).

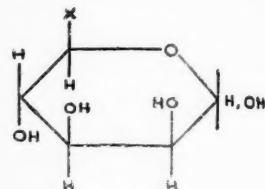
From the above rule three exceptions have been observed. D-xylose which belongs to the D-glucose type does not neutralise *Pisum* lectin, D-digitoxose neutralises *Lotus* lectin although it does not belong to L-galactose or L-talose type, and L-rhamnose has a (weak) inhibiting effect on *Bandeiraea* lectin although it does not belong to D-galactose or D-talose type (Table 1).

The weak inhibition of *Lotus* lectin by N-acetyl-glucosamine, glucose and mannose might be explained by assuming, that someone of these sugars takes a less important position in the H blood group substance.

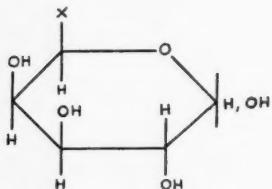
Importance of the Type of the Glycocidic Link (α or β) in Disaccharides. — A monosaccharide often retains its agglutination inhibiting power even when linked to other sugars through the hydroxylic group of the first (in ketoses, second) carbon atom. The type of the link, whether α or β (Fig. 2) seems to be significant. Avery and Goebel (1, 2) have shown that α and β sugar configuration could, in some instances, be differentiated by means of specific immune antibodies. On the basis of the above results it appears that this can be done by means of lectins as well. Thus *Pisum sativum* lectin is inhibited by α -glucosides and *Cytisus sessilifolius* lectin by β -glucosides but not vice versa. The significance of these observations for blood group research will be discussed at a later date.

**D - GLUCOSE TYPE**

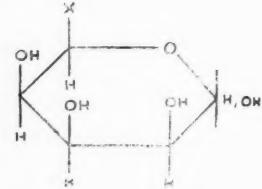
- X = -H, D - XYLOSE
- X = -CH₂OH, D - GLUCOSE
- X = -CH₃, D - QUINOVOSE
- X = -CHOH - CH₂OH, TWO ALDOHEPTOSES

**D - MANNOSE TYPE**

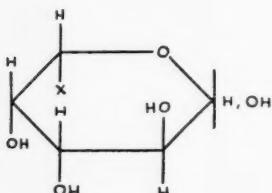
- X = -H, D - LYXOSE
- X = -CH₂OH, D - MANNOSE
- X = -CH₃, D - RHAMNOSE
- X = -CHOH - CH₂OH, TWO ALDOHEPTOSES

**D - GALACTOSE TYPE**

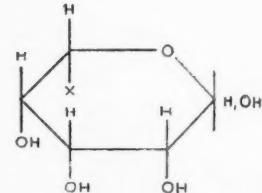
- X = -H, L - ARABINOSE
- X = -CH₂OH, D - GALACTOSE
- X = -CH₃, D - FUCOSE
- X = -CHOH - CH₂OH, TWO ALDOHEPTOSES

**D - TALOSE TYPE**

- X = -H, L - RIBOSE
- X = -CH₂OH, D - TALOSE
- X = -CH₃, 6 - DEOXY - D - TALOSE
- X = -CHOH - CH₂OH, TWO ALDOHEPTOSES

**L - GALACTOSE TYPE**

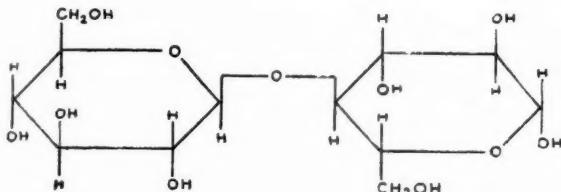
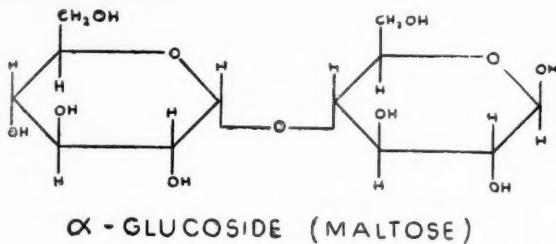
- X = -H, D - ARABINOSE
- X = -CH₂OH, L - GALACTOSE
- X = -CH₃, L - FUCOSE
- X = -CHOH - CH₂OH, TWO ALDOHEPTOSES

**L - TALOSE TYPE**

- X = -H, D - RIBOSE
- X = -CH₂OH, L - TALOSE
- X = -CH₃, 6 - DEOXY - L - TALOSE
- X = -CHOH - CH₂OH, TWO ALDOHEPTOSES

Fig. 1. — The basic structures of some homomorphous sugar series.

The inhibiting effect of melibiose (α -D-galactoside) on the (blood group B specific) *Bandeiraea* lectin is much stronger than that of lactose (β -D-galactoside). This observation is in agreement with the opinion of Kabat and Leskowitz (5) that the active side chain



β -GLUCOSIDE (CELLOBIOSE)

Fig. 2. — The structure of maltose and cellobiose as examples of α -and β -glycosides.

in the B substance has a terminal nonreducing D-galactose linked by a 1-6- α -linkage to another sugar.

D-galactose seems to be a constituent of the «receptor» of the *Crotalaria* lectin, too, but in this case lactose is a more efficient inhibitor than melibiose.

It seems possible, on the basis of the above results, to ascertain the type of the glycosidic link (α or β) of glucose (and perhaps of glucosamine & D-mannose) in disaccharides and some other oligosaccharides of partially known structure.

By means of the lectins of *Bandeiraea* and *Crotalaria* it seems possible to obtain information of the type of D-galactosidic (and perhaps L-arabinosidic, D-talosidic & D-fucosidic) link, even though the differences between α - and β -galactosides are less definite (Table 2) than those between α - and β -glucosides.

If need be, the above determinations can be done with 0.01—1 mgs of sugar, and the solution need not be pure, amino acids and many other sugars, for example, do not interfere. There are other methods of studying the nature of the glycosidic link, but the amounts of sugar needed for enzymatic or polarimetric study are greater, and for polarimetric determination very pure solutions are necessary. The information obtained by chromato-

TABLE 2
THE AGGLUTINATION INHIBITION EFFECT OF OLIGOSACCHARIDES

		Minimum Amount of Sugar (Millimoles per Litre) Inhibiting the Action of			
		<i>Pisum Sativum</i> Lectin	<i>Cytisus Sessilifol.</i> Lectin	<i>Bandeiraea Simplicifol.</i> Lectin	<i>Crotalaria Juncea</i> Lectin
α -Gluco-sides	Maltose ..	1.25	>80	>80	>80
	Sucrose ..	2.5	>80	>80	>80
	Turanose ..	0.6	>80	>80	>80
	Trehalose ..	0.6	>80	>80	>80
β -Gluco-sides	Cellobiose ..	>80	5	>80	>80
	Gentibiose ..	>80	40	>80	>80
α -Galacto-sides	Melibiose ..	>80	>80	0.3	5
	Raffinose ..	>80	>80	0.3	20
β -Galacto-sides	Lactose ..	>80	>80	20	1.25

graphic or infra-red spectrographic studies is limited unless an adequate stock of reference compounds is available.

The sugar specificity of lectins has some resemblance to that of glycosidases. This supports a suggestion of there being some connection between these two vegetable agents. Lectins are not, however, likely to be true glycosidases because (a) they are present in ungerminated seeds and their amount does not increase appreciably after germination (8), (b) there is no correlation in occurrence between lectins and glycosidases in plant species (4), and (c) several purified lectin preparations from dormant seeds have been found incapable of hydrolysing disaccharides which inhibit agglutination (4). (d) Germinated seeds of *Laburnum alpinum* contain a lectin which is inhibited by β -glucosides but not by α -glucosides, yet the extract made from these seeds hydrolyses maltose (α -glycoside) but apparently not cellobiose (β -glycoside) (8).

SUMMARY

Different plant hemagglutinins (lectins) are neutralised by different simple sugars. Their sugar specificity has some resemblance

¹ The tubes contained 2 to 4 agglutinating doses of lectin.

to that of plant glycosidases, e.g. they are able to differentiate between α - and β -glycosides. Because of this capability they can be of use when it is desired to ascertain the type of the glycosidic link in some oligosaccharides of partially known structure. Lectins seem, however, not to be true glycosidases.

ACKNOWLEDGEMENT

The authors wish to extend their thanks to Mr. E. K. Vaughan, Curator, Botanical Garden, University College of Ghana, Achimota, Ghana, who kindly sent them several samples of *Bandeiraea simplicifolia* seeds.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF
HELSINKI

THE BACTERICIDAL ACTION OF THE RARE EARTH METALS

FURTHER STUDIES

by

ALI MUROMA

(Received for publication April 18, 1959)

In the writer's previous paper (1) dealing with the bactericidal action of the salts of rare earth metals, seven metals in this group were not included, *i.e.*, Gd, Tb, Dy, Ho, Er, Tm and Lu. In the present work the bactericidal effect of the chlorides of these seven metals and, in addition, of Y and Yb on two bacterial strains was studied. Performance of the bactericidal experiments and evaluation of the results were similar to those in the preceding work (1, pp. 22—24).

The metallic chlorides used in this investigation were prepared from oxides with a purity over 99 per cent, being in some cases 99.9 per cent. The oxides were the generous gift of Heavy Minerals Co., United States. *Micrococcus pyogenes var. aureus* and *Shigella dysenteriae* were used as test bacteria. The first mentioned was freshly obtained from a routine sample sent for examination, and the latter was a laboratory strain, the same as bacterium No. 18 in the earlier work.

RESULTS

In the experiments in tables 1 and 2 the lowest bactericidal solution of all the salts in 24-hour experiments was 10^{-4} or 10^{-5} molar. During 5 minutes' and 1 hour's exposure the bactericidal

TABLE I
BACTERICIDAL ACTION OF METALLIC SALTS AGAINST *Micrococcus pyogenes var. aureus*

Metallic Salt	Action of Salt Solutions during the Three Exposure Times											
	5 Minutes				1 Hour				24 Hours			
	Molar Concentration of Salt			Molar Concentration of Salt			Molar Concentration of Salt			Molar Concentration of Salt		
10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-7}
GdCl ₃	○				○					○	■	■
TbCl ₃	○				○					○	■	■
DyCl ₃	○				○					○	■	■
HoCl ₃	○				○					○	■	■
YCl ₃					■					○	■	■
ErCl ₃										○	■	■
TmCl ₃	○									○	■	■
YbCl ₃	○				■					○	■	■
LuCl ₃	○	○								○	○	■

■ = Bactericidal action

○ = No experiment made

TABLE 2
BACTERICIDAL ACTION OF METALLIC SALTS AGAINST *Shigella Dysenteriae*

Metallic Salt	Action of Salt Solutions during the Three Exposure Times											
	5 Minutes				1 Hour				24 Hours			
	Molar Concentration of Salt				Molar Concentration of Salt				Molar Concentration of Salt			
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
GdCl ₃	○							○				
TbCl ₃	○	■						○				
DyCl ₃	○							○				
HoCl ₃	○	■						○				
YCl ₃				■				■				
ErCl ₃		■						■				
TmCl ₃		○						○				
YbCl ₃		○	■					○				
LuCl ₃		○	○					○				

■ = Bactericidal action
○ = No experiment made

effect of the salts on *M. pyogenes var. aureus* was weak, whereas they had a fairly marked toxic effect on *Sh. dysenteriae*. The previously described paradoxical effect was seen also in these experiments. Thus tables 1 and 2 give of the bactericidal action of the metallic salts a picture that is on the whole similar to that obtained in the author's earlier experiments (cf. 1, table 3 on p. 26, table 4 on p. 27, and table 19 on p. 35). The results now obtained with YCl_3 and YbCl_3 are also practically consistent with the results of the previous investigation.

On comparison of the bactericidal effect of the various metallic salts it is observed that TbCl_3 and DyCl_3 in table 1, and GdCl_3 , TbCl_3 and DyCl_3 in table 2 had a slower action than the other salts, which had an approximately equally rapid action. Thus HoCl_3 , ErCl_3 , TmCl_3 and LuCl_3 may, with respect to their rate of action, be ranked in the same category with YCl_3 and YbCl_3 .

DISCUSSION

With respect to their ionic radius, the 16 known rare earth metals may be placed in the following order (2):
 $\text{La} > \text{Ce} > \text{Pr} > \text{Nd} > \text{Sm} > \text{Eu} > \text{Gd} > \text{Tb} > \text{Dy} > \text{Ho} > \text{Y} > \text{Er} > \text{Tm} > \text{Yb} > \text{Lu} > \text{Sc}$. The 9 rare earth metals earlier studied by the author were placed in the following three groups in the order of increasing rate of bactericidal action (1, p. 45):

$\text{La, Ce, Pr, Nd} < \text{Sm, Eu, Y, Yb} < \text{Sc}$.

In view of the limitations of the method used, a more detailed classification could not be made, and it therefore was not possible to determine the internal order of the metals in the La, Ce, Pr, Nd group and the Sm, Eu, Y, Yb group. Since this order of increasing rate of toxicity is at the same time the order of decreasing radius of the cations, the assumption was expressed that the rate of bactericidal action of the rare earth metals appears in this way to depend upon the size of the ion and possibly upon other properties of the ion connected with its size (1, p. 46).

On basis of the results obtained in the present work the nine metals now studied can be grouped according to the rate of bactericidal effect into two groups, as follows:

$\text{Gd, Tb, Dy} < \text{Ho, Y, Er, Tm, Yb, Lu}$.

Since Gd, Tb and Dy have a larger ionic radius than the other examined cations, the above mentioned observation concerning a correlation between the ionic radius and the bactericidal effect applies also to the present experiments.

All the seven metals that were not included in the previous investigation (1), *i.e.*, Gd, Tb, Dy, Ho, Er, Tm and Lu, can with respect to ionic radius be placed in the middle group shown in the earlier paper (group Sm, Eu, Y, Yb). However, only four of them — Ho, Er, Tm and Lu — can be included in this group with respect to the bactericidal effect according to the results of the present work. Since in the earlier experiments Sm and Eu had the same rate of effect as Y and Yb, and since Gd, Tb and Dy now were slower than Y and Yb, this circumstance is not in agreement with the presented opinion concerning the correlation with the ionic radius. However, since the present results have been derived from only two bacteria and without test series of Nd, Sm and Eu, conclusions must be drawn with caution.

SUMMARY

In an earlier investigation seven rare earth metals — Gd, Tb, Dy, Ho, Er, Tm and Lu — were not included in the experiments. In the present work the bactericidal effect of these seven metals was studied. The results obtained showed that these metals did not differ unexpectedly in effect from the metals studied earlier. The results were in agreement with the previous observation concerning the inverse correlation of the rate of bactericidal effect and the ionic radius, with the reservation that group Gd, Tb and Dy may form an exception. These three metals probably are comparatively slow in effect in relation to their ionic radius.

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FROM THE MEDICAL CLINIC AND THE DEPARTMENT OF PHYSIOLOGY
UNIVERSITY OF TURKU

SERUM SULPHATE AND ENDOGENOUS SULPHATE CLEARANCE IN RENAL DISEASES

by

KASANEN, A., HÄKKINEN, I. P. P., and HÄKKINEN, L. M.

(Received for publication April 22, 1959)

Inorganic sulphate increases in the blood of patients suffering from renal insufficiency. It may even be 20 times higher than normal (1). This retention shows a very close correlation with non-protein nitrogen (8). Inorganic sulphate has also been regarded as a more sensitive retention test than urea and creatinine (13). On the other hand, individual ethereal sulphate fractions, such as the potassium salt of indoxyl sulphate (indican), increase very readily in renal insufficiency. This fraction has been regarded as the most sensitive indicator of renal disease (2). Several workers consider an elevated serum indican level to be a prognostic sign (6, 10, 11 and 12).

The use of endogenous clearances has increased now that they are considered to correspond most closely to physiological conditions. Creatinine and urea in particular are important tests in diagnosis of renal disease, although urea has many drawbacks. Kasanen (4) has studied earlier the possibilities of using indican clearance in renal insufficiency and found it to be a very sensitive test. It had no additional advantages, however, over for instance creatinine clearance.

The clinical use of sulphate retention and clearance was hampered previously by the relatively cumbersome method employed.

Now that a simpler technique of analysis has been worked out (3). It seemed necessary to make comparative studies between serum sulphate reaction and endogenous sulphate clearance and certain renal functions.

MATERIAL AND METHODS

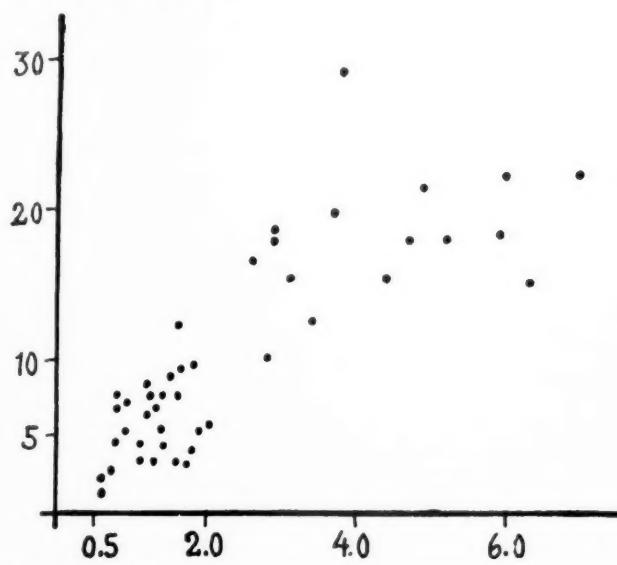
The serum inorganic and total sulphate and the total sulphate clearance was determined in 44 patients with renal disease ranging in severity from quite mild to uremia. The serum creatinine and endogenous creatinine clearance were determined concurrently in the same patients, and likewise the phenolsulphonphthalein excretion and the specific gravity of morning urine. The clearance period was 2 hours. Sulphate determination was based on the precipitation of SO_4 with benzidine and the determination of SO_4 spectrophotometrically with Bachloramilate. The urine SO_4 determination was made with Bachloramilate. The total sulphate in both serum and urine was determined after acid and thermal hydrolysis.

RESULTS

Serum Sulphate. — The level of total sulphate in the serum ranged from 2.8 to 29.3 mg%, mean 8.6 ± 1.15 mg%. The normal values for inorganic sulphate were 2.4—3.3 mg% and for total sulphate 2.7—3.9 mg%. Hence, retention was clearly demonstrable in renal patients. The proportion of inorganic sulphate in total sulphate was c. 60%, range 15 to 96%. The proportion of inorganic sulphate increased with the growth of sulphate retention. In the patients whose serum sulphate exceeded 8 mg% the proportion of inorganic sulphate was $67.1 \pm 7.4\%$ and in the patients whose total sulphate was less than 8 mg% the inorganic sulphate was $54.2 \pm 6.4\%$. In individual cases the greatest part of serum retention occurred as an increase of the inorganic or organic fraction. No correlation was established clinically between the variation of the percentual retention of these different fractions and the nature or severity of the disease.

Fig. 1 shows the correlation between serum sulphate and serum creatinine. The correlation coefficient was $+ 0.66 \pm 0.09$, i. e. highly significant. Serum creatinine was elevated in five cases when the

Serum sulphate in mg %



Serum creatinine in mg %

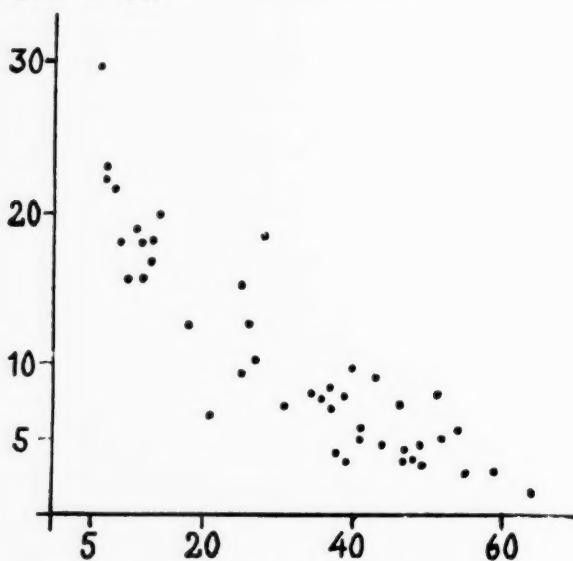
Fig. 1. — The correlation between serum sulphate and serum creatinine.

serum sulphate was normal. In only one case was creatinine normal when the sulphate was elevated. Sulphate retention was no more sensitive than creatinine, rather the contrary.

The correlation coefficient between serum sulphate and phenol-sulphonphthalein excretion was -0.34 ± 0.11 , *i. e.* significant. The test results were contradictory in many individual cases. Phenol-sulphonphthalein excretion was lowered in 7 cases when the sulphate was elevated and phenol red excretion was normal. Phenolsulphonphthalein excretion may consequently be regarded as a more sensitive test than serum sulphate.

The correlation coefficient between serum sulphate and the specific gravity of morning urine was -0.20 ± 0.14 , *viz.* non-significant. The specific gravity of morning urine was a considerably more sensitive renal test than serum sulphate. This is due to the fact that the majority of the patients had pyelonephritis for which a lowered specific gravity is the most sensitive indicator of renal function (5).

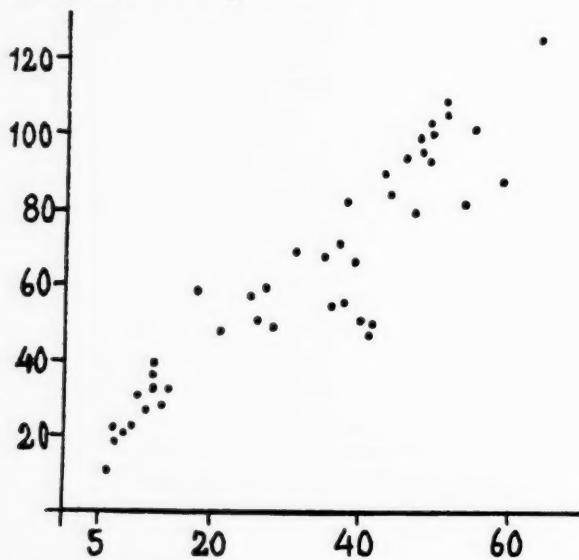
Serum sulphate in mg %



Sulphate clearance in c. c. per min.

Fig. 2. — The Correlation between serum sulphate and sulphate clearance.

Creatinine clearance in c. c. per min.



Sulphate clearance in c. c. per min.

Fig. 3. — The correlation between sulphate and creatinine clearance.

Endogenous Sulphate Clearance. — The normal sulphate clearance was 37 c. c. per min., but when the serum sulphate was elevated the clearance rose to 95 c. c. per min. It has been also demonstrated that the sulphate clearance is about 30% of the creatinine clearance. At normal plasma sulphate levels the sulphate clearance is always less than the creatinine clearance and usually less than the urea clearance (9,6). Normal indican clearance is also of this magnitude, i.e. 41 ± 5 c. c. per min.

The mean of the total sulphate clearance in the material was 33 c. c. per min., variation 6.0—64 c. c. per min. The individual fractions of the inorganic and organic fractions could not be calculated as it was found that the ethereal sulphates of the urine were not stable but disintegrated 50% in about 30 min. We have been unable so far to establish the reason for this phenomenon. The correlation between serum sulphate and endogenous clearance is shown in Fig. 2; the correlation coefficient was -0.69 ± 0.12 . In all instances where the serum showed retention, clearance was lowered. It was consequently impossible to throw additional light on serum determination by the clearance test.

Fig. 3 shows the correlation between sulphate clearance and creatinine clearance; the correlation coefficient was $+0.58 \pm 0.10$, viz. highly significant. Sulphate clearance was generally c. 40% of glomerular filtration, in some individual cases 90%. In no case did it exceed glomerular filtration. In six cases the creatinine clearance was clearly lowered when the sulphate clearance was still normal. In not a single case, on the other hand, did sulphate clearance fall before creatinine.

The correlation coefficient between sulphate clearance and the excretion of phenolsulphonphthalein was $+0.44 \pm 0.11$, i.e. highly significant. Phenol red was clearly more sensitive than sulphate clearance and was already lowered in several cases when the sulphate clearance was still normal. Specific gravity was also a more sensitive test than sulphate clearance.

DISCUSSION

The sulphate clearance was c. 40% of glomerular filtration. There is obviously considerable reabsorption in the tubuli. This hampers the clinical use of the test, as was the case also with uric

acid and indican. The reabsorption percentage obviously grows as the serum level rises and the test is thus not as sensitive as the glomerular function, creatinine clearance and tubular excretion tests, such as phenolsulphonphthalein. Although the material consisted chiefly of pyelonephritis patients with marked tubular lesions, it seems that the other tubular functions, excretion of phenol red and specific gravity, are more sensitive than sulphate clearance.

We hold that sulphate retention and sulphate clearance are not suitable for clinical use on patients with renal disease. Serum creatinine and creatinine clearance, for instance, are physiologically more suitable.

SUMMARY

The inorganic and total sulphate of serum and endogenous sulphate clearance were analysed in a total of 44 patients with renal disease. They were compared with other renal tests. The mean of serum total sulphate was 8.6 mg%, of endogenous sulphate clearance 33 c. c. per min. Serum creatinine was a more sensitive retention test than serum sulphate. Sulphate clearance was c. 40% of glomerular filtration. With the retention of sulphate the clearance value approached glomerular filtration. Creatinine clearance and the excretion of phenolsulphonphthalein were more sensitive tests than sulphate clearance. Sulphate retention and endogenous sulphate clearance are obviously unsuitable for clinical use.

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FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF TURKU

ANTERIOR HYPOPHYSIS-GONAD INTERRELATIONSHIPS AFTER CORTISONE TREATMENT

by

U. K. RINNE* and E. NÄÄTÄNEN*

(Received for publication May 5, 1959)

Clinical investigations have revealed that prolonged cortisone treatment causes disturbances in the function of the gonads. Women have exhibited menstrual irregularities and men a diminution of sexual potency (*e.g.* 6, 9, 10).

Experiments have been conducted with laboratory animals to shed light on the phenomenon, but the results have been exceedingly inconsistent. Some researchers have observed cortisone to reduce the weight of the gonads as well as of the testes and the ovaries and to bring about histological changes in them, indicating diminished activity (*e.g.* 1, 7, 17). Other investigators, however, have noted no specific effect in this respect (*e.g.* 12, 20, 15, 2, 3). On the other hand, it has been asserted that cortisone has spermatogenetic activity, being capable of maintaining spermatogenesis in rats after hypophysectomy and adrenalectomy (13).

The contradictory results of research clearly bring out the fact that the effect of cortisone treatment on the sexual glands is still in many respects an open question. In order to illuminate the problem further, the effect of cortisone treatment on the whole anterior hypophysis-gonad system has been investigated in the present study by noting the histological changes in the gonads and the anterior hypophysis and determining the gonadotrophic hormone content of the hypophysis and of the urine following cortisone treatment.

* Aided by a grant from the Reumaliitto.

MATERIAL AND METHODS

Adult male and female albino rats, altogether 100, were used as test animals.

The animals were divided into eight groups, with between 10 and 20 in each group, the number and sex being indicated on the part of the different groups in Tables 1 and 2. Cortisone acetate* was injected subcutaneously daily in a dosage of 2.5 mg/100g body weight (0.1 ml). A daily injection of 0.1 ml saline was given the controls. All the female rats were at their dioestrus at the beginning of the experiment and were killed at the end of five weeks. The oestrus cycle was followed also during the final week of treatment. The male rats were killed 2, 4 and 5 weeks after treatment started.

From all the cortisone groups excepting the one treated for 2 weeks, urine was collected during a period of four days both before the cortisone treatment and at its end. From the urine collected, the gonatrophins were precipitated with alcohol and an assay was performed with immature female mice.**

The animals were killed quickly by decapitation. The hypophysis, testes and ovaries were removed and weighed in a torsion balance. From all the groups excepting the male rats receiving 5-weeks' cortisone treatment and the corresponding control group, the hypophysis of 7 animals was homogenized in 3 ml of saline. The assay of the gonadotrophic hormone content of the suspension was performed with immature, 3-weeks-old female rats by injecting into them 0.5 ml suspension subcutaneously, twice daily for a period of three days, as has been previously described (8). All the other hypophyses as well as testes and ovaries were fixed for a period of 24 hours in 10% neutral formalin, treated in the usual manner and embedded in paraffin. The organs were cut in a series into 5 μ sections. The testes were stained with hematoxylin-eosin, the ovaries with van Gieson and the hypophyses by means of the periodic acid-Schiff technique.

In the statistical treatment of the results, the t-test was applied. The difference between the means was considered significant by values of $P \leq 0.05$.

* Kindly supplied by Lääke Oy.

** We are indebted to Prof. A. Pekkarinen, the Head of the Department of Pharmacology, University of Turku, for the determinations of the gonadotrophins.

RESULTS

Testes-Table 1 shows that a 2-weeks' cortisone treatment has no appreciable effect on the absolute weight of the testes. On the other hand, among the 4- and 5-weeks' groups, the absolute weight of the testes of the animals receiving cortisone is below that of the controls, the difference in the latter group being statistically significant. The relative weights in all the cortisone groups, however, are greater than among the controls, as the final body weights in the

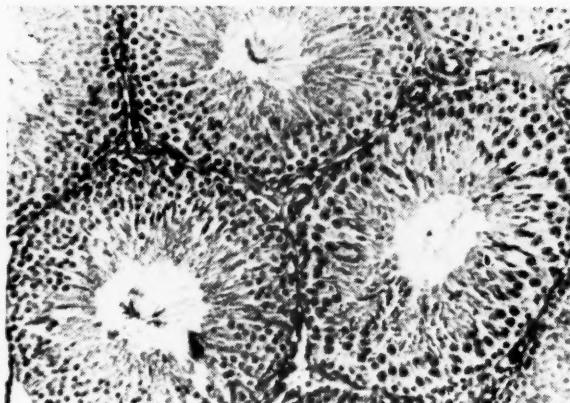


Fig. 1. — Photomicrograph showing a section of the testis of a control rat.
Haematoxylin-eosin $\times 240$.

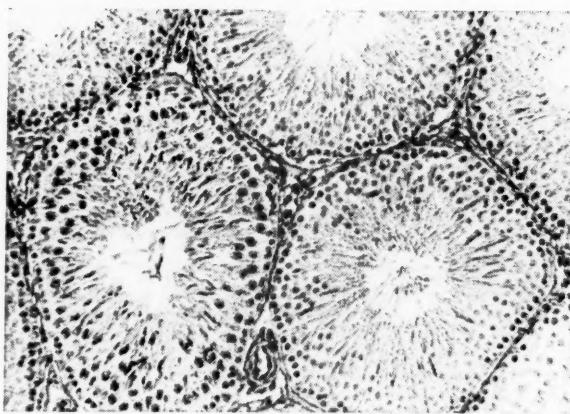


Fig. 2 — Photomicrograph showing a section of the testis of a rat for 5 weeks with cortisone treated rat. Haematoxylin-eosin $\times 240$.

TABLE 1
BODY AND ORGAN WEIGHTS IN MALE CONTROL AND WITH CORTISONE TREATED RATS

Treatment	Duration of Treatment (Wk.)	Number of Animals	Body Weight-gm			Testes			Hypophysis		
			Initial	Final	% Gain	mg	mg/100gm Body Weight	P*	mg	mg/100gm Body Weight	P*
Controls . . .	2	10	181 ± 11.0**	214 ± 8.7	+18	2430 ± 74	1135 ± 52	>0.05	6.3 ± 0.23	2.9 ± 0.20	>0.05
Cortisone . . .	2	10	181 ± 10.1	176 ± 11.8	—3	2438 ± 48	1385 ± 60	>0.05	6.2 ± 0.22	3.5 ± 0.32	>0.05
Controls . . .	4	10	181 ± 12.6	227 ± 13.0	+25	2600 ± 108	1145 ± 42	>0.05	6.9 ± 0.51	3.0 ± 0.35	>0.05
Cortisone . . .	4	20	181 ± 8.3	176 ± 8.7	—3	2442 ± 105	1387 ± 46	>0.05	6.8 ± 0.19	3.9 ± 0.20	>0.05
Controls . . .	5	10	181 ± 9.0	230 ± 11.3	+27	2730 ± 69	1241 ± 79	<0.05	6.1 ± 0.35	2.9 ± 0.19	>0.05
Cortisone . . .	5	10	181 ± 8.5	122 ± 8.7	—33	2440 ± 110	2007 ± 101	<0.05	5.5 ± 0.56	4.5 ± 0.28	>0.05

* P values refer to comparison with corresponding controls in regard to the absolute weight.
 ** Standard error.

TABLE 2
BODY AND ORGAN WEIGHTS IN FEMALE CONTROL AND WITH CORTISONE TREATED RATS

Treatment	Duration of Treatment (Wk.)	Number of Animals	Body Weight-gm			Ovaries			Hypophysis		
			Initial	Final	% Gain	mg	mg/100gm Body Weight	P*	mg	mg/100gm Body Weight	P*
Controls . . .	5	15	153 ± 7.2**	177 ± 5.0	+16	76.0 ± 3.0	42.9 ± 2.9	>0.05	8.4 ± 0.33	4.7 ± 0.20	>0.05
Cortisone . . .	5	15	153 ± 3.0	133 ± 5.1	—13	71.0 ± 3.7	53.4 ± 3.5	>0.05	8.1 ± 0.05	6.1 ± 0.19	>0.05

* P values refer to comparison with corresponding controls in regard to the absolute weight.
 ** Standard error.

cortisone groups were correspondingly smaller than in the control groups.

In the histological examination, it was observed that, taking into account individual variations, there was no distinct difference in respect to the spermatogenetic or interstitial cells between the animals of any cortisone and control groups. (Fig. 1 and 2).

Ovaries-Table 2 shows that the absolute weight of the ovaries of the female rats subjected to cortisone treatment is slightly below that of corresponding controls, but the difference is not statistically significant. On the other hand, the relative weight of the ovaries of animals in the cortisone group is appreciably greater than that among the controls.

In the histological picture of the ovaries, no clear qualitative difference what so ever could be detected between the cortisone and control groups. On the other hand, the animals receiving cortisone appeared to have numerically more corpora lutea than the controls. This is brought out also by Table 3.

It should be mentioned further that in observing the oestrus cycle of female rats, it proved to be normal in both the cortisone and the control groups.

Hypophysis-Tables 1 and 2 show that in both the male and female cortisone groups the absolute weight of the hypophysis is slightly less than among the corresponding controls, but the difference is in no group statistically significant. On the other hand, the relative weight in the cortisone groups is greater than in the

TABLE 3

AVERAGE NUMBER OF PRIMARY, GRAAFIAN AND ATRETIC FOLLICLES AND NORMAL CORPORA LUTEA IN ONE SECTION OF OVARIES OF CONTROL AND WITH CORTISONE TREATED FEMALE RATS

Treatment	Oestrus Cycle*	Number of Animals	Primary Follicles	Graafian Follicles	Atretic Follicles	Corpora Lutea
Controls . . .	oe	5	1.9 ± 0.4	1.0 ± 0.3	5.5 ± 0.5	9.4 ± 0.7
Controls . . .	doe	10	2.3 ± 0.2	0.8 ± 0.2	5.0 ± 0.6	10.0 ± 0.5
Cortisone . . .	oe	6	2.0 ± 0.5	0.7 ± 0.2	5.0 ± 0.6	12.7 ± 0.7
Cortisone . . .	deo	9	2.3 ± 0.3	1.0 ± 0.2	5.0 ± 0.7	10.2 ± 0.7

* Stage of the oestrus cycle at the end of the treatment. oe = oestrus, doe = dioestrus.

** Standard error.

control groups, just as was the case in respect to the testes and ovaries described in the foregoing.

In examining the picture of the anterior hypophysis, there appeared to be somewhat more PAS-positive cells in all the male and female cortisone groups than among the corresponding controls. The shape, size and granulation of the PAS-positive cells varied among both the controls and the animals receiving cortisone, but it appeared as if there were slightly more large, somewhat hypertrophic cells in the cortisone groups than among the controls, particularly in the 4- and 5-week groups. In respect to the granulation of the cells, on account of the relatively great variation, no distinct difference between the test and control animals could be observed.

The Gondotrophic Hormone Content of the Hypophysis and of the Urine. — The gonadotrophic hormone content (GTH) of the hypophysis is shown in Table 4. The absolute weight of the ovaries of the immature assay-rats receiving hypophysis-suspensions from animals treated with cortisone is in all the groups less than that of the corresponding controls, although the differences are not statistically significant. Also the absolute weight of the uterus of

TABLE 4

BODY AND ORGAN WEIGHTS IN IMMATURE FEMALE RATS WITH WHICH THE DETERMINATIONS OF THE PITUITARY GONADOTROPIC HORMONE CONTENT OF CONTROL AND WITH CORTISONE TREATED RATS WAS PERFORMED

Treatment	Donor			Recipient					
	Duration of Treatment (Wk.)	Sex	Number of Animals	Body Weight-gm		Ovary		Uterus	
				Initial	Final	mg	mg/100gm Body Weight	mg	mg/100 gm Body Weight
Controls	2	m*	7	30.0 ± 1.2*	36.8 ± 1.6	23.7 ± 1.7	64.4 ± 3.4	61.9 ± 5.2	167.2 ± 11.2
Cortisone	2	m	7	30.2 ± 1.4	37.8 ± 1.6	22.0 ± 1.6	58.3 ± 3.9	47.4 ± 2.5	126.7 ± 8.0
Controls	4	m	7	31.1 ± 1.2	41.9 ± 1.5	22.7 ± 1.3	54.2 ± 2.9	81.7 ± 4.3	196.3 ± 11.7
Cortisone	4	m	7	31.4 ± 1.0	40.4 ± 1.5	21.8 ± 2.2	53.9 ± 5.0	70.0 ± 5.4	174.7 ± 16.1
Controls	5	f**	7	24.2 ± 1.6	31.7 ± 2.3	14.7 ± 0.9	46.3 ± 0.7	25.7 ± 3.6	80.4 ± 9.8
Cortisone	5	f	7	24.0 ± 1.4	25.5 ± 1.9	12.5 ± 0.8	49.0 ± 2.4	14.6 ± 0.9	58.7 ± 4.3

* Standard error

** m = male

*** f = female

the assay-animals is lower in the cortisone groups than among the controls, the difference being statistically significant in the 2-week male and 5-week female groups. Thus the GTH-content of the hypophysis in the cortisone groups appears to be smaller than among the controls.

In performing the determinations of the GTH excreted into the urine, it was noted that in those cortisone groups where the assay was carried out the weight of the uterus of those assay-mice among which the amount of GTH excreted into the urine was determined after cortisone treatment exceeded by 10 to 20% that of those assay-animals among which, in the corresponding groups, the gonadotrophins excreted before cortisone treatment were determined. Thus cortisone induced a slight augmentation of the GTH excretion into the urine.

DISCUSSION

The study revealed that, even though cortisone treatment induced a slight decrease in the absolute weight of the gonads, it did not occur in nearly the same degree as was true in respect to body weight. Accordingly, the relative weights rose quite noticeably. It has, indeed, been previously demonstrated that the weight of the testes diminishes much less than that of other organs during a period of loss of protein (11), which in the present work was caused by cortisone, as is known.

Although the biological assay used in determining the GTH-content of the hypophysis and the urine are comparatively rough and it is not possible on their basis to say for sure whether a change in the quantity of the follicle-stimulating hormone or/and of the luteinizing hormone is involved, the results can nevertheless be regarded as indicative. Accordingly, the results of the present study indicate that cortisone treatment induced in both the male and female animals slightly augmented GTH excretion, the quantity of GTH having diminished in the hypophysis but increased in the urine. This is in agreement with the observations that there is an increase of GTH excretion into the urine of human beings during cortisone treatment (18, 14), just as in the case of immature male rats it was observed that the quantity of GTH in the hypophysis of cortisone-treated individuals was usually smaller than among the

controls (8). Further evidence of augmented GTH-excretion is provided by the changes noted in the PAS-positive cells of the anterior hypophysis of animals receiving cortisone studies having revealed gonadotrophins to form in them (5, 16). Apparently, the increase in GTH excretion was, however, so slight that it induced no detectable histological changes in the testes. On the other hand, among the female rats a slight increase in the quantity of corpora lutea was observed, this being in agreement with the results previously obtained with immature female rats (4).

Since cortisone causes atrophy of the adrenal cortex through inhibition of ACTH excretion, a further consequence of it is a decrease in the production of sex steroids by this gland (19). Accordingly, the reduced quantity of adrenal sex hormones in the bloodstream might bring about an augmented excretion of GTH. On the other hand, it is possible that with the inhibition of ACTH production by cortisone, the excretion of gonadotrophins and, at the same time of other trophic hormones of the anterior hypophysis might increase.

SUMMARY

The effect of cortisone treatment on the anterior hypophysis-gonad interrelationships was investigated by studying the histological changes in the gonads and anterior hypophysis of adult male and female rats and determining the gonadotrophic hormone content of the hypophysis and of the urine after cortisone treatment.

The cortisone caused a slight decrease in the absolute weight of the hypophysis as well as of the testes and the ovaries, but their relative weights, on the other hand, were appreciably greater than among the controls as a result of the marked decrease in the body weight of the cortisone-treated animals. No specific effect on the histological picture of the testes could be observed after cortisone treatment. On the other hand, the ovaries of cortisone-treated female rats contained slightly more corpora lutea than those of the controls. The animals of the cortisone groups exhibited in addition to a slight hypertrophy a somewhat greater quantity of PAS-positive cells in the anterior hypophysis than the corresponding controls. The gonadotrophic hormone content of the hypophysis was relatively low among the animals receiving cortisone. The

quantity of gonadotrophic hormones excreted into the urine, on the other hand, had slightly increased under the effect of cortisone treatment. The possible mechanisms producing the effects of cortisone treatment on the anterior hypophysis-gonad system are discussed.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY,
UNIVERSITY OF HELSINKI

PAPER CHROMATOGRAPHY OF NITROGEN FREE PHOSPHATIDES OF THE CARDIOLIPIN TYPE¹

by

O.-V. RENKONEN and O. RENKONEN

(Received for publication June 6, 1959)

Phosphatides consisting only of glycerol, fatty acids and phosphoric acid have many interesting biological and biochemical properties (3, 6—9, 12—16). The well-established use of some of these phosphatides in syphilis antigens (1) is also of importance. The known types of natural lipids belonging to this group of phosphatides include the simple phosphatidic acids, phosphatidyl glycerols (2) and the polyglycerol phosphatides, which are represented e.g. by cardiolipin (17).

Chemical, biochemical and serological work on these phosphatides is hampered by the lack of suitable methods for their fractionation. This paper describes two paper chromatographic systems for the separation of well purified preparations of cardiolipin and sitolipin (21) (which is a cardiolipin like phosphatide from wheat germs) into several subfractions.

MATERIALS AND METHODS

Cardiolipin. — The used preparations were purified samples of Na-cardiolipin in ethanol. They were obtained as gifts from Dr. M. Faure, Institut Pasteur (5) and from Dr. M. Macfarlane, Lister Institute (11).

¹ This work was supported in part by a grant from Valtion Luonnon-tieteellinen Toimikunta, Helsinki.

Sitolipin. — The used preparate of Na-sitolipin in ethanol was isolated as described by Uroma and Louhivuori (22). It was obtained as a gift from Dr. M. Tuomioja, State Serum Institute, Helsinki.

Phosphatidyl Serine. — A sample of brain phosphatidyl serine was used. It was obtained as a gift from prof. E. Klenk, Universität Köln.

Monophosphoinositide. — A highly purified sample of monophosphoinositide from wheat germs (4) was used. It was obtained as a gift from Dr. M. Faure, Institut Pasteur. The lipid was used as sodium salt.

Paper Chromatography. — Silicic acid impregnated papers (10) were used. The Whatman No. 1 papers were impregnated as described earlier (18). The impregnated papers were hung for 15 ± 1 minutes before immersion into HCl. The papers were bathed in HCl for 36 minutes. Essentially identical chromatograms were obtained with papers of two different batches.

Lipids were applied on papers as follows: Na-cardiolipin in ethanol, Na-sitolipin in ethanol, Na-salt of monophosphoinositide in chloroform-methanol-water (2:4:1) and phosphatidyl serine in chloroform. The loaded papers were left to dry for 15—20 minutes before the chromatography was started. The development and the staining of the papers with Rhodamine 6G (20) was carried out as described earlier (18).

Di-isobutyl ketone-acetic acid (9:1)* (19) and di-isobutyl ketone-acetic acid (8:2)* (18) were used as developing solvents. The chromatograms were developed until the solvent front had moved about 20—25 cm. This was achieved in about 10 hours.

RESULTS

Tracings of typical paper chromatograms obtained in the two systems used are shown in Figs. 1 and 2.

Fig. 1 shows that the system di-isobutyl ketone-acetic acid (9:1) separates sitolipin in at least four fractions. Also the separation of cardiolipin into two or three fractions has started. The fastest moving fraction of cardiolipin is strongly stained, the middle fraction faintly, and the slowest moving fraction is again moderately strongly stained. — All these fractions of sitolipin and cardiolipin show the characteristical blue-brown colour of anionic phosphatides when stained with Rhodamine 6G and inspected under UV-light. — Fig. 1 shows also that sitolipin does not contain compounds corresponding to the main fraction of cardiolipin. Similarly the three fast moving fractions of sitolipin are not present in cardiolipin.

* Volume ratios.

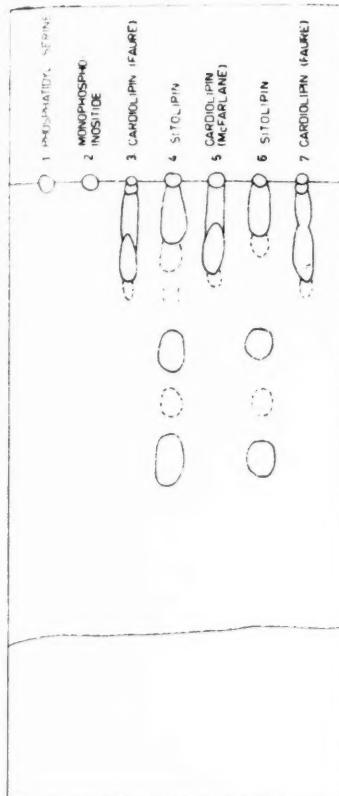


Fig. 1.

Di-isobutyl ketone-acetic-acid (9:1)

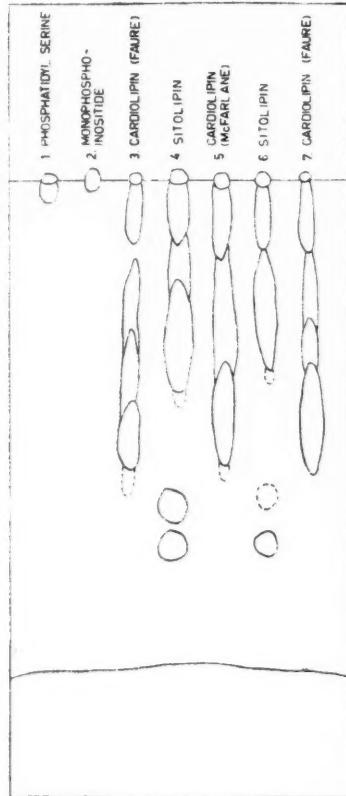


Fig. 2.

Di-isobutyl ketone-acetic-acid (8:2)

Fig. 1 and Fig. 2. — The amounts of the applied lipids are the same in both chromatograms:

- | | |
|-----------------------------------|---------------|
| 1. Phosphatidylserine | ca 25 μ g |
| 2. Monophosphoinositide | ca 12 μ g |
| 3. Cardiolipin (Faure) | ca 20 μ g |
| 4. Sitolipin | ca 20 μ g |
| 5. Cardiolipin (Macfarlane) | ca 25 μ g |
| 6. Sitolipin | ca 10 μ g |
| 7. Cardiolipin (Faure) | ca 20 μ g |

Fig. 2 shows that the system di-isobutyl ketone-acetic acid (8:2) is already too polar for ideal fractionation of the fast moving components, but it separates the fourth, the slowly moving fraction of sitolipin (Fig. 1) in at least two main fractions. Similarly this system separates cardiolipin more clearly into two well stained fractions — the fast moving one and the slowly moving one — with a faintly stained fraction between them. This faintly stained one seems sometimes to be further separated into two different areas. — Fig. 2 also confirms that common compounds of cardiolipin and sitolipin, if any, can be found only in the more slowly moving fractions.

Fig. 1 and 2 show clearly that none of the fractions mentioned is identical with phosphatidyl serine nor with monophosphoinositide, both of which are likely contaminants in cardiolipin and sitolipin.

DISCUSSION

The chemical properties and especially the analytical values reported for both the sitolipin (22) and the two cardiolipin preparations (5, 11) show that they can be regarded as rather pure mixtures of phosphatides which contain only glycerol, fatty acids and phosphoric acid. The mutual similarity of the Rhodamine 6G 'colours' of all the paper chromatographically obtained subfractions therefore suggests that these fractions really represent different types of phosphatides containing only glycerol, fatty acids and phosphoric acid. If this would prove to be the case, it would mean that the paper chromatographic systems described quite likely could be used for fractionation of lipophilic anionic phosphatides in general. It would also mean that cardiolipin is a mixture of different types of phosphatides. Further, sitolipin would then contain new types of natural phosphatides. — We think that the paper chromatographic separation of two similar phosphatides described by Marinett *et al.* (13) supports our findings.

Of practical importance is that many unpolar nonphospholipids have similar paper chromatographic mobilities (19) than the subfractions of sitolipin and cardiolipin described.

Acknowledgements. — We wish to thank also here Drs. M. Faure, Institut Pasteur, M. Macfarlane, Lister Institute and M. Tuomioja, State Serum Institute, Helsinki, as well as Prof. E. Klenk, Universität Köln for gifts of valuable lipid samples.

SUMMARY

Two paper chromatographic systems are described which allow the separation of cardiolipin in two main subfractions. Sitolipin, a cardiolipin like phosphatide from wheat germs, is fractionated by these systems into five different subfractions. All these subfractions of both the cardiolipin and sitolipin are stained with Rhodamine 6G in a manner characteristic to anionic phosphatides. The main subfraction of cardiolipin is not present in the serologically active sitolipin.

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FROM THE MEDICAL DEPARTMENT OF THE PROVINCIAL HOSPITAL, OULU

A SIMPLE METHOD FOR UROPEPSIN DETERMINATION

WITH LITTLE ADJUSTMENT FOR PEPSIN, CATHEPSIN AND TRYPSIN
DETERMINATION FROM URINE, BLOOD, AND OTHER BODY FLUIDS

by

TEPPO VARTIO

(Received for publication May 16, 1959)

Uropepsin literature gives plenty of evidence, that there exists much controversy in the opinions of various authors as to the uropepsin values in different diseases and the clinical use of the uropepsin determination. Many authors have recognized the value of uropepsin determination considering differential diagnosis. It has, according to them, proved useful in the diagnosis of gastrointestinal diseases, notably peptic ulcer and gastric carcinoma (12, 13). In macrocytic anaemias, associated with an achlorhydria not altered by stimulation with histamine, it is a reliable differential test in excluding pernicious anaemia (1, 11). In cases of massive haematemesis, uropepsin values have been useful in the consideration of differential diagnoses (12). The response of uropepsin to hormone stimulation may provide diagnostic indexes to endocrinological conditions (20, 25).

Anyhow, especially in recent studies, the value of uropepsin determination has been the subject of great criticism (5, 8, 15, 17, 18, 29). High values have been obtained in duodenal and gastric ulcer (5, 12, 13, 21) and low values in pernicious anaemia and gastric carcinoma (1, 10, 11, 12, 13), but the values show individual variations and overlapping to such a degree that the uropepsin determination has no or little value in differential diagnosis.

In a recent study Siurala *et al.* (23) stated, that the diagnostic value of uropepsin determination is limited because of the various factors influencing it. However, in case of the extremes of values it might prove useful. High values might speak in favour of the clinical suspicion of duodenal ulcer. Low values, especially if they occur in younger patients with a gastric ulceration, have to be highly suspected of being indications of malignancy. If malignancy is ruled out, the possibility of pernicious anaemia or a state of prepernicious anaemia must be taken into account.

Siurala and Turula (22) showed recently that uropepsin excretion decreased significantly with progressing degrees of gastritis. In patients with severe atrophic gastritis the uropepsin values obtained were of approximately the same class of magnitude as in gastric cancer and pernicious anaemia. »Non-specific» atrophic gastritis was considered to be the most common cause of low uropepsin excretion.

Thus it seems that uropepsin determination, at all events, has some value in clinical use, in estimating the presence of duodenal ulcer, gastric cancer, pernicious anaemia, and »non-specific» atrophic gastritis. Thus it seems justified to introduce a method for uropepsin determination, which, in our experience, is simple enough to be carried on as a »routine» procedure in every-day clinical work. With little adjustments it can be modified for determining also other proteolytic enzymes, cathepsin and trypsin, from urine, blood, and other body fluids.

DESCRIPTION OF THE METHOD

The method is originally that of Buchs (7) for cathepsin determination modified for pepsin and trypsin determination from urine and, with little modifications, also from blood and other body fluids.

Method for Uropepsin Determination. — 0.5 Gm of edestin (Hoffmann-La Roche, Basel) was dissolved in 100 cc. of glycocoll-HCl buffer (85 cc. 0.1 N glycocoll + 15 cc. 0.1 N HCl, the solution was made acid to the methyl orange, *i.e.* pH 3 or less, by adding a sufficient amount of 2 N HCl). 5 cc. of this edestin-buffer + 1 cc. of the urine to be examined were incubated in a waterbath of 37° C for one hour simultaneously with a substrate-blank (1 cc. of water instead of urine). For the determination of the breaking up of the edestin 1 cc. of the substrate-urine was mixed with 8 cc. of 0.5 per cent

gummi-arabicum solution and 1 cc. of 20 per cent sulfosalicylic acid. The same procedure was applied to the substrate-blank. The turbidity caused by the sulfosalicylic acid and stabilized by the gummi-arabicum solution was read in E. E. L.-photometer with blue filter. The amount of the edestin broken up by the urine was calculated from the difference between readings in photometer of the substrate-urine and substrate-blank. The uropepsin activity of the urine was expressed in mg:s of edestin, which was broken up in an hour by the amount of urine excreted in an hour.

Modification for Urocathepsin and Urotrypsin Determination. — The urocathepsin determination was made exactly in the same manner except that the pH of the buffer solution used was 3.3 (85 cc. 0.1 glycocoll + 15 cc. 0.1 N HCl). The urocathepsin activity was expressed in mg:s of edestin broken up in an hour by the amount of urine excreted in an hour.

In the urotrypsin determination, as the edestin was not dissolved in alkaline medium, the protein used was dried human serum. The buffer solution, pH 9.5, was prepared from NH₄CL and NH₃. To obtain a sufficient precipitation, 4 cc. instead of 1 cc. of sulfosalicylic acid was used, and this was taken into account in the final calculation. For the rest the determination was made in the same manner as the foregoing determinations were. The urotrypsin activity was expressed in mg:s of protein broken up in an hour by the amount of urine excreted in an hour.

Modification for Determination of Pepsin, Cathepsin and Trypsin from Other Body Fluids: The examination was made in the same manner as described above, except that the control-tube for the sake of comparison was made in the same manner as the tube for the specimen to be studied, but the precipitation and the reading in photometer was performed immediately. By the reading in photometer the additional turbidity caused by the in many cases originally turbid specimen was taken into account. Thus in these cases the standard-curve was drawn separately for each determination with the help of the control-tube (containing 0.04 per cent of edestin in pepsin and cathepsin determination, and 0.03 per cent human serum protein in trypsin determination) and 0-tube with the specimens own turbidity (made exactly in the same manner as the control-tube but without edestin or human serum protein). The precipitation and the reading in photometer of the 0-tube was also performed immediately. The reading in this 0-tube didn't change noteworthy if the precipitation was made immediately or after being in a 37° C waterbath for one hour, which was verified repeated times. Thus the amount, which the fluid possible breaks up from its own proteins, is minimal. The proteolytic activities were expressed in mg:s of edestin or human serum protein broken up in an hour by one ml. of the specimen to be studied.

The Accuracy of the Uropepsin and Urocathepsin Determination Method: In an earlier study (26) the accuracy of the urocathepsin method was found to be $\pm 2 \times 2.1$ mg edestin/hour, if the determinations were made in triplicates. The accuracy of the uropepsin method was found to be approximately the same.

MATERIAL

The uropepsin excretion was determinated by the method of West *et al.* (30) and by the present method described above from 24 hospital patients without known gastrointestinal complaints or disease, and from 11 patients with gastric ulcer, 10 patients with duodenal ulcer, 10 patients with gastric cancer, and 8 patients with pernicious anaemia. The urine was collected over a period of 24 hours. The blood plasma pepsin, cathepsin and trypsin were determinated by the method described from 11 hospital patients without known gastrointestinal diseases, and from 4 patients with gastric ulcer, 4 patients with duodenal ulcer, 7 patients with gastric cancer, and 5 patients with pernicious anaemia.

RESULTS

The results of uropepsin determination by the method of West *et al.* and by the new method are given in table 1. The distribution of the values with the new method, as appears from table 1, is much smaller than that with the method of West *et al.* In gastric ulcer and in duodenal ulcer both the values were higher on an average than in the material for comparison, and in gastric cancer and pernicious anaemia they were lower on an average. The statistical analysis shows that the difference between the mean values of the material for comparison and those of various disease groups by the method of West *et al.* is not significant in gastric ulcer, almost significant in duodenal ulcer and gastric cancer, and highly significant in pernicious anaemia. By the new method the corresponding difference is almost significant in gastric ulcer and duodenal ulcer, significant in gastric cancer, and not significant in pernicious anaemia.

The results of blood pepsin and cathepsin determinations in various diseases and in cases for comparison appear from table 2. The values of blood plasma pepsin are higher than in the material for comparison in gastric and duodenal ulcer and lower in gastric cancer and in pernicious anaemia. The cathepsin values showed on an average no notable differences in various diseases. The plasma- and urotypsin values were 0 in all cases studied, which is known to be due to the antitrypsin effect of the blood (9, 14, 16).

	METHOD OF WEST ET AL.		NEW METHOD		Difference
	between	Mean	Value	a	

TABLE I. COMPARISON VALUES IN THE MATERIAL FOR COMPARISON AND IN CASES OF VARIOUS DISEASES BY THE NEW METHOD OF WEST ET AL. AND WITH THE NEW METHOD

	Cases No.	Distribution of Values		Mean Value		Difference between Mean Value of a Disease Group and that of the Material for Comparison Statistically	
		Method of West et al. Units/Hour	New Method mg Edestin/ Hour	Method of West et al. Units/Hour	New Method mg Edestin/ Hour	Method of West et al.	New Method
Material for comparison	24	3.5—142.6	0.0—59.2	48.5 ± 7.22	18.8 ± 2.97		
Gastric ulcer	11	1.7—201.4	12.8—88.2	88.7 ± 21.43	41.8 ± 8.33	non significant	almost significant
Duodenal ulcer	10	19.0—254.5	10.1—91.0	114.9 ± 30.52	39.0 ± 8.16	almost significant	almost significant
Gastric cancer	10	0.0—71.7	0.0—18.3	22.9 ± 8.04	6.6 ± 1.99	almost significant	significant
Pernicious anaemia . . .	8	0.0—25.0	0.0—22.7	6.5 ± 2.75	12.4 ± 3.41	highly significant	non significant

TABLE 2. BLOOD PLASMA PEPSIN AND CATEPSIN VALUES IN THE MATERIAL FOR COMPARISON AND IN CASES OF VARIOUS DISEASES

Material for Comparison	Gastric Ulcer			Duodenal Ulcer			Gastric Cancer			Pernicious Anaemia		
	Pepsin mg Edestin/ml	Catepsin mg Edestin/ml	Pepsin mg Catepsin mg Edestin/ml	Pepsin mg Edestin/ml	Catepsin mg Edestin/ml	Pepsin mg Catepsin mg Edestin/ml	Pepsin mg Edestin/ml	Catepsin mg Edestin/ml	Pepsin mg Edestin/ml	Catepsin mg Edestin/ml	Pepsin mg Edestin/ml	Catepsin mg Edestin/ml
0.75	1.45	1.00	0.32	0.60	0.70	0.00	0.00	0.00	0.08	0.08	0.55	
0.35	0.60	2.50	0.47	0.65	0.35	0.60	0.35	0.60	0.02	0.02	0.20	
0.00	0.60	0.60	0.00	1.27	0.35	0.00	1.55	0.17	0.17	0.17	0.40	
0.17	0.50	0.60	0.45	1.02	0.07	0.65	0.15	0.08	0.08	0.08	0.25	
0.35	0.50	mean	mean	0.10	0.37	0.00	0.00	0.00	0.00	0.00	0.30	
0.45	0.32	1.17	0.31	0.88	0.36	0.27	0.27	0.27	mean	mean	mean	
1.12	0.42	0.25	0.07	0.25	0.07	0.27	0.07	0.07	0.07	0.07	0.34	
0.42	0.25	0.80	0.17	0.80	0.17	0.27	0.27	0.27	mean	mean	mean	
0.25	0.70	0.37	0.07	0.25	0.07	0.27	0.07	0.07	0.07	0.07	0.35	
mean	0.48	mean	0.47	mean	0.35	mean	0.35	mean	mean	mean	mean	

DISCUSSION

In recent years the uropepsin determination method of West *et al.* (30) has attained increased use. This method is based on the caseinisation effect of the activated pepsinogen in urine. The time required for caseinisation is determinated in fresh homogenized milk in acetate buffer (pH 4.9) by means of a stopwatch. The time in seconds required to reach the caseinisation point and the volume of urine used in the determination are logarithmically related. This method is very simple, needs little supplies, and is a time-saving procedure. The disadvantage of this method is the difficulty to determine exactly the point of the caseinisation of the milk, which requires great experience, and easily can cause failures especially in the determination of very low or high values. Thus in general the distribution of the values by this method is very great. Besides the time of caseinisation is dependent on the quality of the milk used, which depends on the season, source, age etc. Baur (4) has criticized this method strictly and says that divergencies are caused in the results obtained by this method because the caseinisation is a very complex phenomenon. The latter consists of at least two phases which overlap each other: 1. The converting of caseinogen to casein undepending of calcium-ions, and 2. precipitating of casein as a calcium-salt. This precipitation begins a rather long time before the visibility of the caseinisation. The number and quality of factors which influence the quickness of this reaction are unknown.

The method of Anson (2), Anson and Mirsky (3), and Bucher (6), and its modifications, *e.g.* that of Gray *et al.* (12), which are based on the determination of the amount of tyrosine-like substances, which the proteolytic enzyme to be studied breaks up from a substrate protein, usually hemoglobin substrate, lack the disadvantages of the method of West *et al.* but are more complex and time-consuming, and are thus less suitable for a »routine» clinical use.

The method presented here avoids the disadvantages mentioned above and is simple enough to be carried on as a »routine» procedure in clinical use. Compared with the method of West *et al.* it produces at least as good results as the latter method, and, according to this material, somewhat better results in cases of gastric ulcer and in gastric cancer (table 1). In pernicious anaemia the results

were worse than those obtained with method of West *et al.* (table 1). This might be due to the fact, that, according to Mirsky *et al.* (19), there exists a definite amount of proteolytic activity at pH 1.5 to 3 in blood plasma, which is extragastric in origin and is found in the plasma e.g. after total gastrectomy. It is possible that this proteolytic activity has particularly appeared in these cases of pernicious anaemia. The distribution of the values with the present method was much less than with the method of West *et al.*, and this might cause the greater significance of the mean values in various disease groups by the method described.

The results of determination of plasma pepsin by the present method in various diseases agrees with the results of Spiro *et al.* (24), who used a modification of the Anson's (2) method. According to the present study there was no dependance of plasma pepsin and cathepsin values of each other, as expressed in table 2. This agrees with the results of Mirsky *et al.* (19), who found in plasma of healthy subjects a proteolytic enzyme system, which was active at pH 3.5 to 4 and was independent of the presence of stomach. This reflects itself also in the proteolytic activity of urine, so that the uropepsin and urocatepsin values are independent of each other, as shown by the present writer in an earlier investigation (26).

The method described has been earlier applied by the present author to the investigation of the three proteolytic enzymes in cerebrospinal fluid (27) and in pleural and peritoneal fluids in various pathological conditions (28).

SUMMARY

A simple method for uropepsin determination, with little adjustment for pepsin, cathepsin and trypsin determination from urine, blood and other body fluids, is described. The method has been compared with that of West *et al.* and some applications of the method to clinical use are presented. The advantages and disadvantages of earlier methods compared to the present method are discussed.

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C REACTIVE PROTEIN IN BACTERIAL MENINGITIS

by

ELLI JANSSON, LIISA JALAVA, and ODD WAGER¹

(Received for publication July 25, 1959)

In a previous study (1) it was shown that practically no C reactive protein (CRP), measurable by the antiserum test, was present in the circulation of patients with serous meningitis or paralytic poliomyelitis. Of 74 serum specimens deriving from a total of 22 patients, only three gave a positive test for CRP, and in one serum specimen only the presence of CRP possibly was due to the disease process in the central nervous system.

The differential diagnosis between serous and bacterial meningitis may sometime be difficult in the earliest phase of the illness, particularly when adequate laboratory facilities are not available. Even under optimal conditions, however, diagnostic difficulties are not rare. The total and differential cell count in the cerebrospinal fluid may not be characteristic enough to ascertain the clinical diagnosis, and often enough a preceding antibiotic therapy may have confused both the clinical and laboratory findings. Important decisions, however, must be made within a few hours following the admission of the patient to the hospital. In the case of bacterial meningitis antibiotics in massive doses must be given as early as possible, whereas no antibiotics at all are needed or even advisable in the case of serous meningitis of viral etiology.

¹ Aided by a grant from the Sigrid Jusélius Foundation.

The studies to be described were undertaken with the purpose of gaining information on the possible serviceability of the CRP antiserum test as a complementary aid in the early differential diagnosis between bacterial and serous meningitis. The main question to which an answer was sought in this study was, whether or not CRP is detectable in the early stage of bacterial meningitis.

METHODS AND RESULTS

The test for the CRP was performed as described before (1), by the use of a commercial anti CRP rabbit immune serum (Schieffelin & Co., New York). According to this method, the final semi-quantitative readings were recorded after the capillary tubes had been kept in the refrigerator overnight. A preliminary reading, however, can easily be taken already a few minutes after the reagents have been brought into contact with each others in the capillary tube. This is best accomplished in a dark room by viewing the tubes against a dark background with the light falling from one side. Thus, the presence of the CRP in the circulation of the patient can be detected within less than an hour once the bleeding has been taken, and a qualitative result made available to the clinician in the very earliest phase of the hospitalization.

The study was carried out between July 1957 and May 1959. A total of 93 patients with meningitis were studied, 86 of them before the end of 1958.

The first bleeding for the CRP test was mostly taken within a few hours following admission of the patient to the hospital, and the subsequent ones on the 2., 3., 7., and 14. day of hospitalization.

Out of the total of 93 patients, the final diagnosis in 30 cases was bacterial and in 63 cases serous meningitis. A number of additional cases had to be excluded, because they proved to be those of some other illness than meningitis, which was initially suspected.

Data on the 30 patients with bacterial meningitis are shown in the table. It can be seen that the causative bacterium was cultured from the cerebrospinal fluid in 23 cases, whereas a direct microscopy revealed it in 11 instances only. In 7 cases the diagnosis was based on other than bacteriological laboratory findings and on the clinical picture.

No.	Age (years)	Sex	SIR	W.C.G	Cell Count	Neutrophiles (per cent)	Smear	Culture	The Day of Hospitalization
1.	2.	3.	4.	5.	6.	7.	8.	9.	10.

Case No.	Recorded No.	Age (years)	Sex	SR	WCC	Cell Count	Neutrophiles (per cent)	Smear	Culture	The Day of Hospitalization				
										1.	2.	3.	7.	14.
1	4768/57	1.2			4	5,900	2,229	96	gr+ diplococci	4+	4+	4+	4+	—
2	807/58	56			64	16,300	7,000	90	—	2+	4+	3+	3+	—
3	1317/58	44			76	7,000	500	65	—	3+	3+	—	—	1+
4	1583/58	9			101	17,000	>10,000	100	—	4+	4+	—	—	—
5	3824/58	39			15	6,300	>10,000	99	—	4+	3+	2+	2+	—
6	4044/58	32			18	23,100	3,300	>50	gr+ diplococci	4+	4+	3+	3+	—
7	4097/58	0.4			61	31,200	3,083	98	—	5+	5+	1+	2+	—
8	4826/58	60			41	13,700	1,550	75	gr+ diplococci	5+	4+	4+	3+	2+
9	4420/58	44			22	8,200	>10,000	100	—	3+	4+	3+	—	—
10	2200/59	57			45	16,800	2,667	98	—	4+	4+	3+	3+	1+
11	2443/59	40			9	6,100	8,360	97	—	—	—	—	—	—
12	368/57	1.2			62	10,700	4,590	90	—	—	—	—	—	—
13	2479/58	1.2			66	12,100	1,980	87	gr— rods	3+	3+	2+	2+	—
14	3873/58	0.6			60	4,200	1,136	100	—	5+	5+	5+	5+	—
15	3929/58	3			61	15,900	10,000	98	—	—	—	—	—	—
16	4985/57	41			28	18,700	2,021	97	—	—	—	—	—	—
17	1691/58	2			89	8,400	5,930	85	—	—	—	—	—	—
18	2729/58	3			59	10,100	2,560	82	gr— diplococci	—	—	—	—	—
19	4385/58	37			91	11,100	909	39	—	—	—	—	—	—
20	3333/57	63			70	17,500	952	95	—	—	3+	3+	4+	—
21	3442/57	58			30	10,700	>10,000	99	—	2+	2+	2+	2+	—
22	5135/57	0.5			64	14,300	2,581	98	—	—	5+	5+	5+	—
23	298/58	35			76	11,800	1,824	90	—	—	4+	4+	3+	3+
24	634/58	64			25	8,000	1,525	96	—	—	1+	2+	2+	1+
25	88/59	70			75	16,500	944	80	—	—	3+	3+	4+	5+
26	630/59	31			80	8,900	3,917	92	—	—	4+	4+	5+	1+
27	1938/58	18			26	4,900	380	57	—	—	1+	1+	2+	1+
28	3641/58	42			30	10,100	150	73	—	—	4+	4+	4+	—
29	4080/58	32			16	6,800	112	88	—	—	2+	3+	2+	2+
30	1266/59	34			42	6,800	2,480	95	—	—	3+	3+	3+	—

Note. — The first Sedimentation Rate (SR), White Cell Count (WCC), and data on the cerebrospinal fluid given by the first lumbar puncture are recorded in the table

— At least the following antibiotics had been administered to the patients before their admission to the hospital. Penicillin to patients No. 3, 5, 7, 20, 22, 23, 24, and 27, Aureomycin to patients No. 2 and 13, Sulfa-penicillin to patient No. 16.

— Patients No. 11, 16, 25, 28, and 30 died.

The test for the CRP was consistently positive in specimens taken on the first, second, and third day, whereas 10 out of a total of 25 specimens taken on the seventh day gave negative results.

It should be pointed out that in addition to bacterial meningitis four patients had some other illness which *per se* might have been responsible for the CRP in the circulation. These conditions were pneumonia (case No. 20), endocarditis (Case No. 16), otitis (case No. 11), and cancer of the stomach (case No. 25).

In 50 out of the total of 63 cases of serous meningitis the test for the CRP was consistently negative at repeated bleedings. In five of the CRP negative cases the causative agent probably was mumps virus. One patient had herpes zoster suggesting an etiological rôle of zoster virus in this case. Echo 9 virus was isolated from the stool of three and from the cerebrospinal fluid of one patient. From the stool of 7 patients a non-typed or non-typable cytopathogenic agent was found. In all, virus isolation from stool was attempted in 46 instances, out of which 36 gave negative results. Poliomyelitis virus could not be isolated in a single case.

In 13 out of the total of 63 cases of serous meningitis the test for the CRP was positive on one or more occasions, the first day test being positive in 11 cases. In all, the test for CRP was positive in 26 specimens out of a total of 268 specimens taken from patients with serous meningitis. Eight of the 13 CRP positive patients had some additional condition which might have been responsible for the occurrence of the CRP in their circulation. These conditions were pharyngitis (3 cases), tracheitis, stomatitis, bronchitis, urethritis and pregnancy. An additional patient, heavily treated with antibiotics before admission to the hospital, may have been a case of bacterial meningitis. In the four remaining cases no possible other explanation than serous meningitis itself could be suspected. In one of these cases the serous meningitis was caused by Leptospira *sejroe*.

DISCUSSION

The studies described justify the conclusion that the inflammatory reaction associated with bacterial meningitis generally causes an appearance of CRP in the circulation in the early stage of the illness. All of the 75 serum specimens taken from 30 patients

during the first three days of hospitalization contained amounts of CRP detectable by the antiserum test. Already within a week following hospitalization the CRP had disappeared from the circulation of 10 patients out of 25 patients examined.

As has been found in a previous work and confirmed in the present work, the inflammatory reaction associated with serous meningitis does not generally cause any appearance of CRP in the circulation. If the results obtained in both of these studies are combined, then it occurs that out of a total of 342 serum specimens deriving from 85 patients only 18 deriving from 15 patients contained measurable CRP. The first bleeding taken from these 15 CRP positive patients contained CRP in 11 instances. Out of these 15 patients, however, the positive CRP test in 10 cases may have been caused by some other accompanying condition rather than the serous meningitis. It is possible, furthermore, that some of the CRP positive cases of serous meningitis in fact have been cases of bacterial meningitis suppressed by a preceding antibiotic therapy given before admission to the hospital.

It can thus be concluded that CRP generally appears in the circulation in the early phase of bacterial meningitis, whereas this is the case only exceptionally in serous meningitis. It seems to us that the test for the CRP may prove itself a useful complementary aid in the early differential diagnosis between these two clinical entities. It is simple to perform even without any laboratory facilities, and a preliminary qualitative result is obtained within less than an hour after the specimen has been taken from the patient¹.

Among the patients with bacterial meningitis there were four cases of tuberculous meningitis. All 13 bleedings taken from these patients contained CRP. The differential diagnosis between serous and tuberculous meningitis quite often is difficult. The number and morphology of cells found in the cerebrospinal fluid do not give any certain clues, the sugar content in the tuberculous cerebrospinal fluid is by no means always low, and no bacteriological support can be expected until some weeks have elapsed. Therefore, if it can be shown with a greater material that tuberculous meningitis regularly causes an appearance of CRP in the circula-

¹ In the June issue of the American Journal of Clinical Pathology, Hyland Laboratories advertised reagents for a new slide method for the CRP determination which gives the test results in two minutes.

tion, then the CRP test no doubt will become a useful new tool in the differential diagnosis between tuberculous and serous meningitis. A study along these lines is in progress.

SUMMARY

Thirty patients with bacterial meningitis were investigated for the occurrence of C reactive protein (CRP) during the early course of the illness. The test for the CRP was consistently positive in specimens taken on the first, second, and third day of hospitalization, whereas 10 out of 25 specimens taken on the seventh day gave negative results.

The observation, made in a previous work, that the inflammatory reaction associated with serous meningitis does not generally cause any appearance of CRP in the circulation, was confirmed.

Among the patients with bacterial meningitis there were four cases of tuberculous meningitis. All 13 bleedings taken from them contained CRP.

It was concluded that the test for the CRP may prove itself useful in the early differential diagnosis between serous and bacterial meningitis, and even more so between serous and tuberculous meningitis.

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FROM THE MUNICIPAL BACTERIOLOGICAL LABORATORY AND KIVELÄ HOSPITAL,
HELSINKI

THE PERSISTENCE OF THE AGGLUTINATION ACTIVATING FACTOR (AAF) IN THE CIRCULATION

A NINE YEAR STUDY OF TWENTY-SEVEN PATIENTS

by

KIMMO AHO, JUHANI KIRPILÄ, and ODD WAGER¹

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Within the last ten years extensive studies of the Waaler-Rose hemagglutination test and its relation to various aspects in rheumatoid arthritis have been published. A good review on this subject has been given by Ziff in 1957 (8). Relatively little, however, is known of the changes of the Waaler-Rose titer of individual patients in various phases of their illness. Pike *et al.* (5) followed 54 patients for four to twenty-nine months and did not find any consistent relation between changes in the clinical symptoms and the hemagglutination titer. Similar observations have been reported by Ball (3) and Alexander & de Forest (2). According to de Forest *et al.* (4) the test became negative in the serum of 10 patients out of a total of 15 experiencing »dramatic grade I clinical remissions».

To our knowledge no studies of longer than three years duration have been published. Thus, it was felt by us worth while to attempt a re-examination of patients first examined by one of us in 1949, *i.e.* nine years ago (7). In the present work such a re-examination of 27 patients was performed and the results compared with those given by the first examination in 1949.

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MATERIAL AND METHODS

For the intended study in all 62 patients were selected from the case material of 1949 (7), according to following criteria.

- Cases classified as typical rheumatoid arthritis with definitely positive Waaler-Rose test (28 patients).
- Cases classified as typical rheumatoid arthritis with definitely negative Waaler-Rose test (16 patients).
- Cases classified as »non-specifically positive», i.e. those with definitely positive Waaler-Rose test but no clinical evidence of rheumatoid arthritis (19 patients).

All of the patients of groups A. and B. and most of the patients of group C. had been under treatment at the Kivelä hospital when first examined. Out of the total of 62 patients, some had died, some moved out of Helsinki since 1949. Thirty-six of them, however, still were residing in Helsinki, and out of these we succeeded in contacting 27 persons. Clinical and serological data of these 27 patients are accounted for in this paper. Of these patients 13 belonged to group A., and seven to each of the groups B. and C.

The clinical activity and the stage of the rheumatoid process were evaluated following the criteria proposed by Steinbrocker *et al.* (6). All of the patients classified as typical rheumatoid arthritis fulfilled the criteria required for the diagnosis of »definite rheumatoid arthritis» by American Rheumatism Association.

The Waaler-Rose Test. — For the determination of the *Differential Agglutination Titer* (DAT), the technique described by Wager (7) was followed with minor modifications. Instead of a 0.5 per cent sheep cell suspension as used in the original technique, a 0.25 per cent suspension was used, because the latter in preliminary studies proved to give a more precise endpoint reading, particularly when a bottom reading technique was applied. Furthermore, the incubation of the tube series in a water-bath of 37°C was omitted, as it was shown by extensive trials (1) that the final result was not affected by this. A DAT of 16 or more was considered positive.

In addition to the determination of the DAT, as described above, each patient serum after removal of the normal sheep cell agglutinins was examined for agglutination of sensitized sheep cells. For this purpose two parts of serum to be tested was absorbed with one part of packed sheep cells by keeping the mixture over-night in the ice-box. An absorption control tube was always included when determining the *Sensitized Sheep Cell Titer* (SSCT). A titer of 128 or more was considered positive.

RESULTS AND DISCUSSION

The results are summarized in the accompanying table. Although it is true that the serological data of 1949 and 1958, due to differences

TABLE
CLINICAL AND SEROLOGICAL DATA ON 27 PATIENTS EXAMINED IN 1949 AND
AGAIN IN 1958

Case No.	Age in 1958 (Years)	Sex	1949		1958		1949		1958		Remarks	
			DAT	DAT	SSCT	Act.	Stage	Act.	Stage			
Group A	1	28	♀	1024	32	1000	++	II	—	II	Bedridden 1958 —→—	
	2	68	♀	512	256	4000	+	II	+++	IV		
	3	74	♀	512	64	500	+	II	—	IV		
	4	67	♀	512	64	1000	+	II	—	IV		
	5	50	♂	256	256	1000	+	II	++	II		
	6	62	♀	256	512	2000	+	II	++	III		
	7	66	♀	256	32	128	—	I	—	II		
	8	44	♀	256	64	500	—	II	+	III		
	9	53	♂	256	16	500	++	II	—	III		
	10	69	♂	128	64	250	—	I	+	II		
	11	64	♀	128	32	1000	—	II	—	IV		
	12	47	♀	128	256	4000	+	II	++	III		
	13	58	♀	256	256	8000	++	II	+	IV		
Group B	14	64	♀	4	32	250	+	II	+++	IV		
	15	38	♀	4	8	500	—	I	—	III		
	16	69	♀	4	2	16	—	I	—	II		
	17	59	♀	4	4	32	—	II	—	III		
	18	66	♀	2	4	500	—	III	—	III		
	19	49	♀	2	4	64	—	I	—	I		
	20	59	♀	2	4	16	—	II	+++	III		
							Clinical Diagnosis in 1949					
Group C	21	53	♀	256	4	32	Appendicitis acuta?					
	22	35	♀	256	32	2000	Myocarditis p. febr. rheum. Insuff. valv. mitr.					
	23	62	♀	128	1		Acrodermatitis atrophicans Herxheimer					
	24	37	♀	128	64	250	Febris rheumatica Hepatitis					
	25	50	♀	128	32	128	Arthralgia					
	26	67	♀	64	8	1000	Struma nodosa					
	27	71	♂	32	4	64	Fractura pectrochanterica l. sin.					

DAT = Differential Agglutination Titer

SSCT = Sensitized Sheep Cell Titer following absorption of the normal sheep cell agglutinins

Group A. and B. included only patients with definite rheumatoid arthritis,
Group C. included patients with "non-specifically positive" Waaler-Rose tests in 1949.

The evaluation of the activity and stage in 1949 was carried out in 1958 and was based on data of the hospital records from 1949, whereas the evaluation of the status in 1958 was based on the examination of the patient itself.

in the technique and other conditions under which the tests were performed, are not fully comparable, the data still allow reasonably safe conclusions as to the presence or absence of measurable Agglutination Activating Factor (AAF) in the circulation of the patients on two occasions separated from each others by nearly ten years.

From the table it can be seen that all of the patients with typical rheumatoid arthritis and a positive Waaler-Rose test DAT value in 1949 (group A.) still contained in their circulation amounts of the factor measurable by the DAT technique as well as by the SSCT technique. A definite clinical progression of the disease process had taken place in 11 cases out of the total of 13. In five cases this progression had led to a severe invalidity, the patients being more or less permanently bedridden.

The situation among the patients of group B. was different. Only one out of the total of seven patients with typical rheumatoid arthritis and a negative Waaler-Rose test in 1949 now contained in her circulation amounts of rheumatoid factor measurable by the DAT technique. By the SSCT technique, however, three patients of group B. gave a positive test result. Although a definite progression of the disease process had taken place in 6 out of the total of 7 patients, none of them had become as permanently crippled as the five patients of group A.

Among the patients with typical rheumatoid arthritis (groups A. and B.) no definite correlation between the Waaler-Rose test and the clinical pattern of the disease process was discernible. It is remarkable, however, that five patients with a particularly malignant character of the rheumatoid arthritis all belonged to group A., consisting of patients who already in 1949 had a positive Waaler-Rose test. This may be suggestive of a prognostic value of the Waaler-Rose test.

Out of the total of seven patients who in 1949 had shown a »non-specifically positive» DAT value (group C.), only three showed a positive test result on re-examination by the DAT technique. Whether or not this greater tendency to reversal from positive to negative, as compared with the typical cases of group A., is significant and possibly reflects a qualitative difference between »specifically» and »non-specifically» positive Waaler-Rose results, cannot be decided from these scarce data. It is interesting to note,

furthermore, that none of the patients of group C. during the nine years of observation had developed any definite symptoms of rheumatoid arthritis.

SUMMARY

Twenty patients with definite rheumatoid arthritis and seven patients with other illnesses and a »non-specifically positive» Waaler-Rose test in 1949 were re-examined following an interval of nine years. All thirteen patients with rheumatoid arthritis and a positive Differential Agglutination Titer (DAT) value in 1949 still gave a positive test result on re-examination, whereas only one out of seven patients with rheumatoid arthritis and a negative DAT value in 1949 gave a positive test result on re-examination.

A definite clinical progression of the rheumatoid disease process had taken place in 17 out of the total of 20 patients with definite rheumatoid arthritis. In five cases this progression had led to a severe invalidity, and all these patients belonged to those who already in 1949 had a positive DAT value. This may be suggestive of a prognostic value of the Waaler-Rose test.

Out of the total of seven patients who in 1949 had shown »non-specifically positive» Waaler-Rose test, only three showed a positive DAT value on re-examination. None of the patients of this group had developed rheumatoid arthritis during the observation period of nine years.

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FROM THE DEPARTMENT OF MEDICAL CHEMISTRY, UNIVERSITY OF
TURKU, FINLAND

ARTEFACTS FROM METHIONINE IN ACID HYDROLYSIS AND CHROMATOGRAPHY

by

J. PIKKARAINEN and E. KULONEN

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Bailey has shown that about 20% of methionine is lost during hydrolysis, if large amounts of carbohydrates are present (1). The destruction of pure methionine in 20% hydrochloric acid has been studied by Osono, Mukai and Tominaga (7), who observed the formation of new ninhydrin-positive spots, especially if the mixture contained certain sugars. Dent (3) reported that methionine was oxidized during phenol chromatography to methionine sulphoxide so rapidly that the spots were of almost equal strength. Koloušek, Liebster and Babicky (5) claimed that this happens also in butanol and that 0.2% thioglycol does not protect the methionine against oxidation.

An incidental observation of non-predicted spots prompted us to study the effect of the acid strength and other conditions of the treatment on the oxidation products and the influence of the chromatography.

EXPERIMENTAL

Reagents. — Acetic acid, ammonia and hydrochloric acid were *pro analysi* (E. Merck AG.), *n*-butanol, crystallized phenol, and usual amino acids were »Laboratoriumspräparate» (E. Merck AG.). Methionine sulphoxide, dl-*a*-amino-*n*-butyric acid, *allo*threonine and cysteic acid (Light & Co., Ltd.), were of »nondesignated» grade as also ninhydrin (Nutritional Biochemicals Corporation). dl-Methionine (E. Merck AG.) was purified by crystallization from aqueous ethanol.

Chromatography. — A frame for simultaneous ascending chromatography of 8 sheets was used (2). All the runs were carried out at the room temperature (21—22°C.) on Whatman No. 1 paper. The first solvent was always butanol-acetic acid-water mixture (4:1:5) of Partridge (9). This run was repeated after drying the paper in the air. The second solvent was water-saturated phenol (with 1% v/v of concentrated ammonia). After the phenol run the papers were dried at +70°C. in the hood and sprayed with 0.2% solution of ninhydrin in 96% ethanol. The papers were kept for the colour development for 15—30 min. at 70°C., except those chromatograms intended for densitometric evaluation, which were allowed to develop at room temperature for 12 hours. For identification several mixed and pure substances were chromatographed.

Treatment with Acid. — For qualitative determination of the destruction products 0.05 mg. amounts of methionine were dissolved into 0.5—2.0 ml. of hydrochloric acid (5.7 N or 11.4 N), the tubes closed by melting and kept at +105°C. for 4, 12 or 24 hours. The samples were diluted about ten-fold with water and the hydrochloric acid was removed by repeated evaporation on water bath. The residues were dissolved into 80% ethanol and applied as spots. One series was made using a reflux condenser instead of the sealed tubes to explore the effect of open air.

In the quantitative evaluation 0.025 mg. amounts of methionine were used and the spots in one-dimensional (butanol mixture as the solvent) chromatograms recorded with the densitometer (Joyce, Loebl & Co., Ltd., Model 3 SR). The areas of the peaks were estimated by a planimeter and the percentage proportion of each peak calculated.

Effect of Chromatography. — One-dimensional chromatograms with butanol mixture were made of 0.01 and 0.02 mg. amounts of original and recrystallized methionine dissolved 10% propanol-water or in 0.1 N hydrochloric acid. Two-dimensional chromatograms were also made using phenol as the first solvent.

RESULTS

Qualitative. — In chromatograms several spots were observed (Fig. 1 and 2). Aspartic acid, methionine sulphoxide and methionine were identified in mixed chromatograms. We are not quite certain on the spot No. 1 assigned to homocysteic acid, which was not available for comparison, but the location corresponds to a homologue of the cysteic acid.

The spot No. 3 could be some unknown derivative of methionine, but presumably a second spot of the aspartic acid (as a salt). The spot No. 4 could not be identified with threonine, glycine, serine or *allo*threonine. At this location there should appear none of the common natural or synthetic amino acids (8). Often a faint spot corresponding to alanine was noted.



Fig. 1.

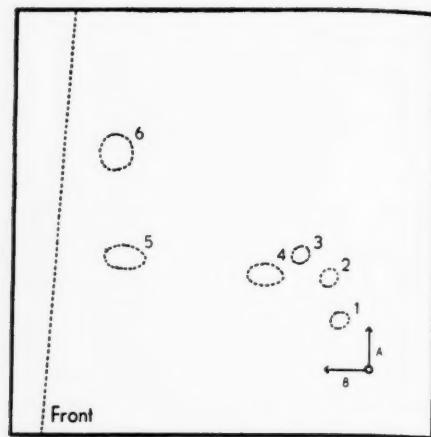


Fig. 2.

Fig. 1. — Effect of various conditions. Solvent butanol, direction upwards. Left: 0.025 mg. recrystallized methionine kept in 5.7 N hydrochloric acid at +105°C. for 4 hours. Right: otherwise similar but treated in 11.4 N hydrochloric acid for 24 hours.

Fig. 2. — Ninhydrin-positive spots from methionine boiled under reflux condenser for 24 hours in 5.7 N hydrochloric acid. Solvents: A = butanol-acetic acid-water, B = water-saturated ammoniacal phenol. Tentative identification: 1 = homocysteic acid, 2 and 3 = aspartic acid, 4 = unknown, 5 = methionine sulphoxide, 6 = methionine.

TABLE I
PRODUCTS OF METHIONINE DURING ACID TREATMENT AT 105°C. (PERCENTAGE PROPORTION OF THE COLOUR BY NINHYDRIN IS PRESENTED)

Product	4 hours	12 hours	24 hours
In 5.7 N Hydrochloric Acid:			
Homocysteic Acid	0 %	1.1 %	8.9 %
Aspartic Acid	1.9 %	3.4 %	4.7 %
Unknown	1.9 %	3.7 %	6.1 %
Methionine Sulphoxide	28.4 %	28.0 %	49.3 %
Methionine	67.8 %	63.8 %	31.0 %
In 11.4 N Hydrochloric Acid:			
Homocysteic Acid	5.1 %	2.3 %	5.5 %
Aspartic Acid	1.9 %	6.5 %	5.8 %
Unknown	2.3 %	8.8 %	6.5 %
Methionine Sulphoxide	20.6 %	23.8 %	23.6 %
Methionine	70.1 %	58.6 %	58.6 %

Conditions of Acid Treatment. — Quantitative changes appear in the Table 1. The amount of destruction increases during the treatment. Of the investigated conditions most favorable is the 5.7 N hydrochloric acid in sealed tube and worst is the strong acid under reflux condenser. Methionine sulphone was observed only at 4 hours treatment in 11.4 N hydrochloric acid in sealed tube and in 5.7 N hydrochloric acid in open conditions. The amount of hydrochloric acid did not influence the results. Aspartic acid was not observed when recrystallized methionine was used. The original sample did not contain aspartic acid.

Chromatography. — A slight oxidation to methionine sulphone was observed only in two-dimensional chromatograms, when phenol was used as the first solvent, but not in other conditions mentioned above.

DISCUSSION

The radiochemical degradation of methionine has been studied in detail (5, 6). Three main routes of destruction were observed: deamination, demethylation and oxidation of the sulphur. In this study the formation of methionine sulphoxide seems to be the main way of destruction, but methionine sulphone is encountered as a transient state only. Demethylation leading to aminobutyric acid seems not important. The side-effects of chromatography were almost nil and are thus not inevitable.

There is no reason to believe that findings would become simpler when other substances are present. It may be pointed out that the formation of aspartic acid did not occur when recrystallized sample was used. In many conditions of acid hydrolysis, especially if carbohydrates are present, there appear oxidative trends (4).

SUMMARY

The destruction of methionine during acid treatment was studied. Seven ninhydrin-positive spots were observed and some of them identified and determined quantitatively.

Acknowledgement. Financial support from the Jenny and Antti Vihuri Foundation and Sigrid Jusélius Foundation is gratefully acknowledged.

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EQUILIBRATION OF SODIUM AND POTASSIUM BETWEEN INTRAPERITONEALLY INJECTED DEXTRAN AND BLOOD PLASMA IN NORMAL AND ADRENALECTOMISED RATS

by

CHRISTINA BRINK, RAILI ESILÄ and M. J. KARVONEN

(Received for publication April 25, 1959)

The intraperitoneal route is widely used in administering various pharmaca to experimental animals. The possibility of using artificial ascites for relieving the work of kidneys in uraemia has also been studied (7). Exchange of cristalloids evidently occurs through the peritoneal membrane in both directions. In »natural» ascites, the concentrations of bicarbonate, chloride, sodium and hydrogen ions are in a Gibbs-Donnan equilibrium with those in plasma (5).

In small animals, taking blood samples for electrolyte determinations is sometimes technically difficult, and the withdrawal of blood of itself brings about a rise of potassium in the plasma. It would therefore be advantageous, if sampling fluid introduced into the peritoneal space could be used for indicating plasma electrolyte levels. Buxton *et al.* (1) have actually published a method, in which cellophane bags containing a small volume of Krebs's solution are introduced in the abdomen; the sodium and potassium levels in the bags equilibrated with the plasma in less than 20 minutes in cats and guinea pigs.

The method of Buxton *et al.* requires surgical interference. The intraperitoneal injection of a colloid is a much simpler procedure.

However, information has not been available on the rates of equilibration of electrolytes in such a preparation. This problem was taken under study in rats. In order to find out whether the adrenals have any effect on the rate of equilibration, experiments were made also on adrenalectomized rats.

METHODS

Adult rats weighing approximately 220 g were used. The experiments on adrenalectomised rats were made 3 days after the operation. They were given standard diet, without adding sodium chloride. For the injections, dextran iso-osmotic with plasma («Macrodex», Pharmacia Ab, Uppsala) was employed. According to the declaration, it contains sodium 0.9 ± 0.1 g per 100 ml (from 154 to 170 m.eq.per l) and no potassium. The molecular weight of dextran is $80\,000 \pm 10\,000$ (4). In half of the experiments, potassium was added to make its content appr. 10 m.eq.per l. The actual sodium and potassium content of each injected sample was determined.

20 ml of dextran was injected intraperitoneally in each rat. This causes no signs of irritation, as also observed by Schubert (7). Samples from the intraperitoneal fluid were taken with a needle at suitable intervals; the fluid flowed freely from the needle.

Blood samples were taken from the tail vein before injection and at the end of the experiment, under light aether anaesthesia. The adrenalectomised rats stood the anaesthesia poorly and fewer blood samples could be taken from them.

The sodium and potassium determinations were made with a Kibbeb Zonen flame photometre. We are indebted for the determinations to Miss Anna-Liisa Puranen, M.A.

RESULTS

Fig. 1 shows the results of experiments in which dextran without added potassium was injected into normal rats. At the end of the experiments, the concentration of sodium in the peritoneal fluid was about 15—20 m.eq. per l lower than in plasma. Potassium, on the other hand, rose within appr. half an hour to the same levels as in plasma. However, the correspondence of potassium between plasma and peritoneal fluid was not absolute.

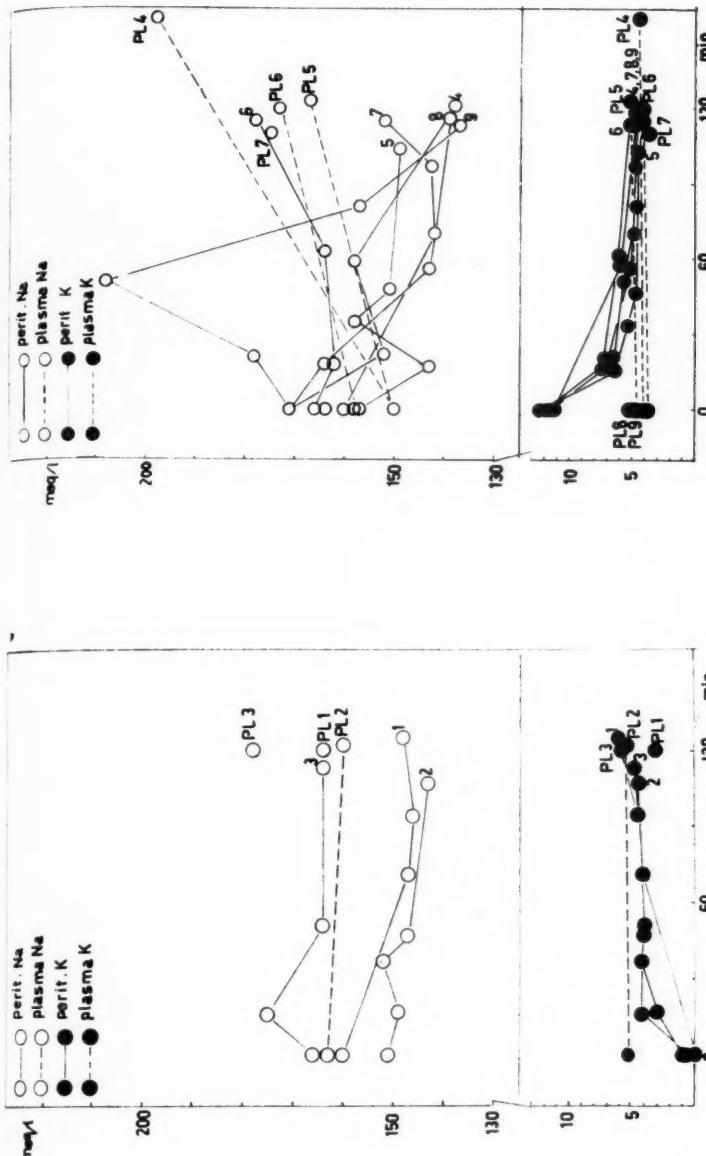


Fig. 1. — Peritoneal and plasma Na and K levels after an intraperitoneal injection of dextran without added K to normal rats.

The numbers refer to Table 1.

Fig. 2. — Peritoneal and plasma Na and K levels after an intraperitoneal injection of dextran with added K to normal rats.

The results of experiments in which dextran with potassium was injected, are shown in Fig. 2. The changes in the sodium followed an irregular course. In five of six experiments, the concentration of sodium had fallen below the starting values in 2 hours. Sodium in plasma, on the other hand, rose. The concentration of potassium fell gradually during the experiment, and at the end of two hours it had reached values rather close to those in plasma. Table 1 shows the changes in plasma potassium and the end levels of peritoneal potassium in detail.

In the adrenalectomised rats the plasma potassium level was to start with distinctly higher than in normal rats, as was to be expected (Table 1). After injection of dextran without added

TABLE 1

POTASSIUM LEVEL IN PLASMA AT THE BEGINNING AND AT THE END OF THE EXPERIMENT, AS COMPARED WITH THE FINAL LEVEL IN THE PERITONEAL FLUID

Experiment No.	Potassium m.eq. per Litre			
	Plasma		Peritoneal	
	Beginning	End		
<i>Adrenals intact</i>				
<i>Without K</i>				
1	...	3.1	6.1	
2	5.2	5.4	4.6	
3	..	5.8	4.8	
<i>With K</i>				
4	4.1	4.5	4.6	
5	4.6	5.2	4.5	
6	3.8	5.2	5.2	
7	..	3.7	4.2	
8	5.2	..	4.6	
9	4.7	..	4.6	
<i>Adrenalectomised</i>				
<i>Without K</i>				
10	6.2	9.8	6.5	
11	5.9	
12	6.4	10.6	6.2	
<i>With K</i>				
13	9.9	..	6.9	
14	7.1	
15	7.7	

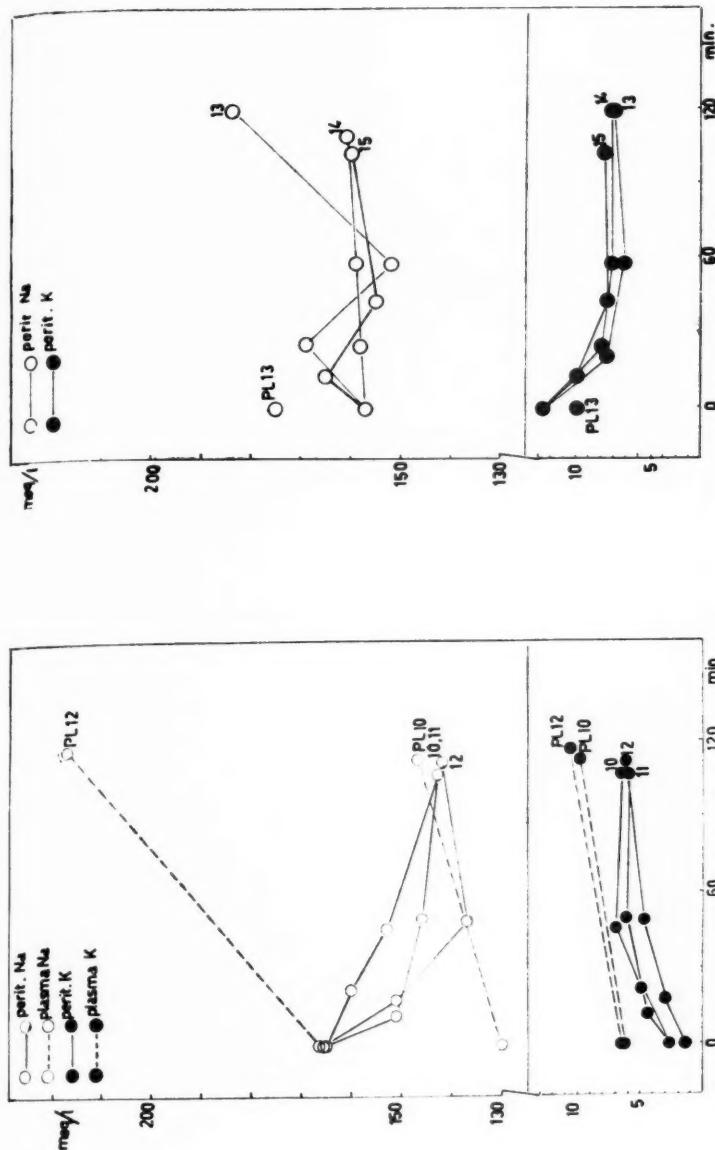


Fig. 3.—Peritoneal and plasma Na and K levels after an intraperitoneal injection of dextran without added K to adrenalectomised rats.

Fig. 4.—Peritoneal Na and K levels after an intraperitoneal injection of dextran with added K to adrenalectomised rats.

potassium (Fig. 3), potassium in plasma nevertheless rose. For sodium, the results were similar to those obtained in rats with intact adrenals.

In the adrenalectomised series in which potassium was added to dextran (Fig. 4), the content of potassium in the peritoneal fluid decreased similarly as in the series of normal rats, remaining, however, on a higher level, as corresponding to the evidently high plasma potassium level. Sodium differs in this group from the majority of other experiments in the sense that the final sodium level in the peritoneal fluid exceeded that in the injected dextran.

DISCUSSION

In the present experiments, a large amount of isotonic and isoosmotic solution — 20 ml — was injected into the peritoneal cavity. For a rat weighing 220 g. the total amount of water may be assessed at 150 ml of which the extracellular volume is appr. 45 ml. From the intraperitoneal fluid, colloids are absorbed into lymphatics, whereas exchange of cristalloids occurs also across the capillary membranes (6, 8).

Plasma injected into peritoneal cavity of rats, guinea pigs or rabbits is absorbed more rapidly than isotonic sodium chloride (2, 3). In the present series, the rate of the absorption of neither dextran nor the cristalloids was followed. However, the wide variations in the sodium concentration of the peritoneal fluid indicate that sodium and water were removed from the peritoneal space at different rates. For sodium, only extracellular space is available, and consequently its absorption results in a marked increase of the plasma level. A corresponding increase would, of course, also result from a transfer of water to the peritoneal space and consequent haemoconcentration. However, the contribution of haemoconcentration to the increases of plasma sodium is not likely under the conditions of the present experiments.

After adrenalectomy, more »leakage« of sodium into the intracellular space may be expected to occur. However, if this was the case, it did not essentially affect the results. Whether the relatively high final levels of peritoneal sodium in the experiments presented in Fig. 4 have anything to do with adrenalectomy, is not known.

For sodium, the practical conclusion is that an injection of dextran leads to a marked rise of the plasma sodium, whereas sodium in the peritoneal fluid varies irregularly and independently of the plasma level.

Extra potassium injected with the dextran disappears causing only a minimal rise in the plasma potassium level (Fig. 2, Table 1). It is obviously taken up into the intracellular space. After adrenalectomy, this mechanism may be less effective, as the level in the peritoneal fluid tends to be higher in experiments with added potassium (Table 1).

When no potassium was added to the injected solution, potassium from the intracellular space was evidently mobilised, since the potassium level in the peritoneal space rose without any decrease in the plasma level (Fig. 1). Adrenalectomy did not affect this change (Fig. 3). The rise of the plasma potassium level in the adrenalectomised rats even when no potassium was added (Fig. 3, Table 1) may have been due to a «leakage» of intracellular potassium due to the critical condition of the animals.

In rats with intact adrenals, the level of potassium in the peritoneal space settles within two hours rather close to that in plasma. However, the agreement is not absolute. This is not surprising, since no general equilibrium is reached; *e.g.* the gradient in the sodium level between plasma and peritoneal fluid may exert a Donnan-effect on the potassium. For demonstrating marked changes in plasma potassium level, the present method may have practical value, but it cannot replace direct analysis in accurate work.

SUMMARY

20 ml isotonic, iso-osmotic dextran (»Macrodex») was injected intraperitoneally to normal and adrenalectomised rats. In about half of the injections, no potassium was added, and the concentration of potassium was made to appr. 10 m.eq. per litre in the rest. The concentrations of sodium and potassium in the fluid were observed with the aid of repeated samples during two hours. Plasma sodium and potassium levels were determined at the beginning and at the end of the experiments.

The injections caused a marked rise of the plasma sodium level. In most experiments the sodium concentration in the peritoneal

fluid decreased, following an irregular course. There was no tendency to equilibration of the plasma and peritoneal sodium level. No systematic effect could be ascribed to adrenalectomy.

In one to two hours, the *potassium* level in the peritoneal fluid was at approximately the same level as in plasma, independently of the potassium content of the injected dextran solution. After adrenalectomy, the plasma levels were higher and similarly also the final levels in the peritoneal fluid.

It was concluded that sampling an intraperitoneal dextran space may give an approximate indication of the plasma potassium level, but none of the plasma sodium level.

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AUS DER MEDIZINISCHEN UNIVERSITÄTSKLINIK, TURKU, FINNLAND

ÜBER AGGLUTINATIONSÄHNLICHE PHÄNOMENE IM
FINGERBEERENBLUT NACH ZUGABE VON GLYCYL-
GLYCIN UND OXYPROLIN. KLINISCHE
BEOBACHTUNGEN¹

von

J. L. KALLIOMÄKI, E. RUBINSTEIN und S. J. VIIKARI

(Der Schriftleitung eingegangen am 22. Juli 1958)

Früher sind interessante Beobachtungen über agglutination-sähnliche Phänomene im Blut nach Zugabe von Peptiden und Aminosäuren dargestellt worden (1). Von den neun erforschten Aminosäuren veranlassten DL-Threonin, L-Oxyprolin, Methionin und Glycin und weiter von neun Peptiden DL-Alanyl-Glycin, L-Leucyl-Glycin, Glycyl-Glycin, Glycyl-Glycyl-Glycin und DL-Leucyl-Glycyl-Glycin ein stärkeres oder schwächeres Agglutinationsphänomen der Rotzellen, wenn ihre auf pH 7 eingestellten 10—1%igen Wasserlösungen zum Fingerbeerenblut hinzugegossen wurden. Das untersuchte Material waren sechs gesunde Personen. Die stärkste Agglutination wurde mit den glycinhaltigen Peptiden erhalten. Bei Zusatz von NaCl im Molekularverhältnis 1 : 1 blieben alle Reaktionen aus. Ein Agglutinationsphänomen wurde auch nicht mit gewaschenen Blutkörperchen zustandegebracht. Für die Entstehung des Phänomens dürften also wenigstens die elektrischen Ladungsdefizite und die Bluteiweise eine gewisse Bedeutung haben. Für die letzterwähnte Sache spricht weiter der Umstand, dass der isoelektrische Punkt aller Aminosäuren und

¹ Diese Arbeit konnte dank der Unterstützung die Sigrid Jusélius-Stiftung durchgeführt werden.

Peptiden, die eine Agglutination veranlassten, in die Gegend zwischen pH 5,5 und 6,2 fiel oder grob genommen in die Gegend, in welcher auch die isoelektrischen Punkte der Bluteiweisse liegen.

EIGENE VERSUCHE

Mit Hilfe unserer Versuche wollten wir ermitteln, in welchem Masse das erwähnte Agglutinationsphänomen von verschiedenen Krankheiten und operativen Massnahmen abhängig wäre. Wir haben zum Objekt ein Peptid, nämlich Glycyl-Glycin, und von den Aminosäuren L-Oxyprolin gewählt. Von beiden wurde eine auf pH 7 eingestellte Wasserlösung gemacht. Namentlich interessierte es uns, ob zwischen verschiedenen Krankheitsgruppen Unterschiede hinsichtlich der Glycin-Glycin- und Oxyprolin-Verdünnungen, mit denen die erwähnte Agglutination noch zustandegebracht werden konnte, wahrzunehmen wären. Von Glycyl-Glycin wurden 2, 1 1/2, 1, 0,75, 0,50 und 0,25%igen Wasserlösungen gemacht. Mit diesen Verdünnungen wurde das Fingerbeerenblut von zehn gesunden, elf an verschiedenen akuten Infektionen leidenden und acht an chronischer Polyarthritis leidenden Personen sowie ferner von zehn Herzinfarktspatienten, zehn Karzinompatienten und vier Ulkuspatienten untersucht. Bei den Karzinom- und Ulkuspatienten wurden die Proben nach der wegen der Grundkrankheit ausgeführten Operation am folgenden Tage noch einmal gemacht. Im ganzen wurde also bei 53 Personen das Verhalten des Fingerbeerenbluts zu verschiedenen Glycyl-Glycin-Lösungen untersucht.

Von Oxyprolin wurden 2, 1 und 0,5%igen Verdünnungen gemacht und damit das Fingerbeerenblut von sieben an chronischer Polyarthritis leidenden und fünf gesunden Personen untersucht.

Die mit Glycyl-Glycin erhaltenen Resultaten sind aus Tabelle 1 und die mit Oxyprolin erhaltenen aus Tabelle 2 ersichtlich.

Wie Tab. 1 zeigt, ist das mit Glycyl-Glycin zustandegebrachte Agglutinationsphänomen sehr konstant. Eine 1 1/2%ige Lösung führte in allen Gruppen eine Agglutination herbei. Die mit der 1%igen Lösung erhaltene Agglutination war variabel, und einige reagierten schon negativ. Wenn man die akuten Infektionen und die chronische Polyarthritis zu einer Gruppe und die Infarkte, die Karzinome und die Ulzera zur anderen grossen Gruppe vereint, so gab es in der ersten, am ehesten also infektionsähnlichen

TABELLE 1

DIE AGGLUTINATIONPHÄNOMENE MIT 2.1 1/2, 1.0, 75.0, 50 UND 0.25 %:IGEN GLYCYL-GLYCIN LÖSUNGEN

Diagnose	Patient	Glycyl-Glycin Lösungen					
		2 %	1 1/2 %	1 %	0.75 %	0.50 %	0.25 %
Gesund	1.	++	++	++	-	-	-
	2.	++	++	+	-	-	-
	3.	++	++	-	-	-	-
	4.	++	++	+	-	-	-
	5.	++	++	+	-	-	-
	6.	++	++	+	-	-	-
	7.	++	++	+	-	-	-
	8.	++	++	+	-	-	-
	9.	++	++	-	-	-	-
	10.	++	++	+	-	-	-
Bronchitis	11.	++	+	-	-	-	-
Nephritis ac.	12.	++	++	+	-	-	-
Pyelonephritis ac.	13.	++	+	+	-	-	-
	14.	++	++	+	-	-	-
	15.	++	++	+	-	-	-
	16.	++	++	+	-	-	-
Pneumonia	17.	++	++	++	-	-	-
	18.	++	++	+	-	-	-
Pleuritis	19.	+++	+++	+++	+	+	-
	20.	+++	+++	+++	+	+	-
	21.	++	++	+	-	-	-
Polyarthritis chr.	22.	++	++	-	-	-	-
	23.	+++	+++	+++	-	-	-
	24.	++	++	++	-	-	-
	25.	++	++	+	-	-	-
	26.	++	+	+	-	-	-
	27.	+	+	+	-	-	-
	28.	+	+	+	-	-	-
	29.	+	+	+	-	-	-
Infarctus cordis	30.	++	++	-	-	-	-
	31.	+	+	+	-	-	-
	32.	++	++	+	-	-	-
	33.	+	+	-	-	-	-
	34.	++	++	+	-	-	-
	35.	++	++	+	-	-	-
	36.	++	++	+	-	-	-
	37.	++	++	+	-	-	-
	38.	++	++	+	-	-	-
	39.	++	++	+	-	-	-
(a coli	40.	++	++	-	-	-	-
ventr.	41.	++	++	-	-	-	-
	42.	++	++	+	-	-	-
	43.	++	+	+	-	-	-
	44.	++	++	+	-	-	-
mammae	45.	++	++	+	-	-	-
	46.	++	++	+	-	-	-
	47.	++	++	+	-	-	-
	48.	++	++	+	-	-	-
	49.	+	++	-	-	-	-
Ulcus duodeni &	50.	++	++	+	-	-	-
ventr.	51.	++	++	-	-	-	-
	52.	++	+	-	-	-	-
	53.	++	++	+	-	-	-

— keine Agglutination

++ +++++ verschiedene starke Agglutinationen

+- unsichere Agglutination

TABELLE 2
DIE AGGLUTINATIONPHÄNOMENE MIT 2.1 UND 0.5 %IGEN OKSYPROLIN LÖSUNGEN

Diagnose	Patient	Oksyprolin Lösungen		
		2 %	1 %	0.5 %
Gesund	1.	+	—	—
9	2.	+	—	—
9	3.	+	—	—
9	4.	+	—	—
9	5.	+	—	—
Polyarthritis chr	6.	+	—	—
9 9	7.	+	—	—
9 9	8.	+	—	—
9 9	9.	+	—	—
9 9	10.	+	—	—
9 9	11.	+	—	—
9 9	12.	+	++	—

Gruppe, zwei Fälle von neunzehn ($10 \pm 7\%$), die negativ auf eine 1%ige Lösung reagierten, und in der letzteren nichtinfektionsähnlichen Gruppe 17 Fälle von 24 ($71 \pm 9.5\%$), die auf dieselbe Verdünnung negativ oder unsicher reagierten. Der Unterschied zwischen den Gruppen ist also demnach $61 \pm 12\%$. In der Infektionsgruppe fallen besonders drei Pleuritisfälle auf, bei denen das Agglutinationsphänomen noch mit einer 0.75%igen Glycyl-Glycin-Lösung zustandegebracht werden konnte.

Bei den Karzinom- und Ulkuspatienten wurden die Agglutinationsproben einen Tag nach der wegen der Grundkrankheit ausgeführten Operation wiederholt, die Resultate stimmten aber mit den vor der Operation erhaltenen Resultaten völlig überein.

In Tab. 2 sieht man, dass die bei den an chronischer Polyarthritis Leidenden mit Oxyprolin gemachten Agglutinationsproben hinsichtlich der Resultate nicht von den bei den gesunden Personen erhaltenen Resultaten abwichen. Leider hatten wir wegen Mangels an Oxyprolin keine Möglichkeiten zu mehreren Verdünnungen und weiteren Serien.

DISKUSSION

Unsere Resultate scheinen darauf hinzuweisen, dass bei den infektionsähnlichen Krankheiten die mit Glycyl-Glycin-Wasserlösungen von dem Fingerbeerenblut erhaltenen Agglutinationsphänomene durchschnittlich mit stärker verdünnten Glycyl-Glycin-Lösungen herbeigeführt werden konnten als bei den nicht-infektionsähnlichen Krankheiten. Dies lässt vermuten, dass einige für Infektionen typische Eiweiss- oder Ladungsveränderungen möglicherweise etwas mit dem Agglutinationsphänomen zu tun haben könnten, worauf schon in der am Anfang erwähnten Forschung hingewiesen worden ist. Weitere Forschungen sind an unseren Klinik im Gange.

ZUSAMMENFASSUNG

- 1) Die Autoren haben das Agglutinationsphänomen untersucht, das die verschiedenen auf pH 7 eingestellten Glycyl-Glycin- und Oxyprolin-Wasserlösungen im Fingerbeerenblut von gesunden und an verschiedenen Krankheitstypen leidenden Personen, sowie vor und nach Operationen herbeiführten.
- 2) Eine 1½%ige Glycyl-Glycin-Lösung veranlasste bei allen Untersuchten ein Agglutinationsphänomen. Bei den infektionsähnlichen Krankheiten wurde mit einer 1%igen Lösung das Agglutinationsphänomen in $90 \pm 7\%$, aber in den nicht-infektionsähnlichen Krankheiten nur in $29 \pm 9.5\%$ erhalten. Der Unterschied ist $61 \pm 12\%$. Mit einer 0.75%igen Lösung wurde die Agglutination noch bei allen drei Pleuritispatienten und unsicher bei einem Polyarthritis chr.-Patienten erzielt.
- 3) Die mit den Glycyl-Glycin-Lösungen vor und nach Operationen gemachten Agglutinationsproben gaben übereinstimmende Resultate.
- 4) Mit den Oxyprolin-Lösungen wurden Agglutinationsproben nur bei Polyarthritis chr.-Patienten und gesunden Personen gemacht. Die Resultate waren nicht abweichend.

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DIFFERENCE IN THE AGGLUTINABILITY OF ROOSTER AND HEN ERYTHROCYTES BY THE TICK-BORNE ENCEPHALITIS VIRUS

by

AIMO SALMINEN¹

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When Sabin and his collaborators (13, 12) in 1950 first discovered the hemagglutinating capacity of arthropod-borne viruses, they stated that regular and reproducible results were obtained only with red cells derived from newly hatched chicks, while erythrocytes from older chicks and chickens gave lower titers, irregular or negative results. Hemagglutination studies have been carried out thereafter with various members of the arthropod-borne group using erythrocytes from one-day-old chicks (10, 8, 18, 5, 7, 16, 2, 17, 14). In 1957, Porterfield (11) reported that also erythrocytes from adult domestic geese were agglutinable and considerably more convenient to obtain. Goose erythrocytes were recently adopted for routine hemagglutination tests with the arbor viruses (6). Positive results have also been obtained in some cases with sheep (13, 12, 14), pigeon (8) and duck (11) erythrocytes.

Based on experiments with a strain of the Russian tick-borne encephalitis virus, it will be presented in this paper that, even though adult hen erythrocytes in the majority of cases gave negative or poor results, erythrocytes from adult roosters were without exception agglutinated to a high titer and are therefore suitable for hemagglutination studies.

¹ Aided by a grant from the Sigrid Jusélius Foundation

MATERIAL AND METHODS

The hemagglutinating capacity of erythrocytes from 26 adult roosters, 24 adult hens and 20 one-day-old chicks was studied with the tick-borne encephalitis virus antigen at different pH's and temperatures. For comparison, erythrocytes from two adult geese (*Anser domesticus romanus*) were also included in the series. The breed of chickens was White Leghorn and the blood samples were collected at one and the same poultry² in the spring.

The virus strain used in the experiments was a strain of the Russian biphasic tick-borne encephalitis virus »Belyanchikov« which was isolated by the working group of Prof. A. A. Smorodintsev (15) from the blood of a patient near Leningrad in 1949. Since then the strain has been transferred intracerebrally in mice, the number of passages being unknown. The strain was received at this laboratory from Leningrad on April 27, 1958.

Suckling mouse brain tissues extracted by acetone and ether according to the method of Casals *et al.* (3, 1, 2) were earlier used as antigens (14). Later on it was found that a strong and useful hemagglutinating antigen could be obtained by a simple alkaline aqueous extraction of brain material (4, 6). Such antigens were stable indefinitely at +4°C and showed no inhibition zone in strong concentrations at optimum pH's. The experiments reported here were carried out with this type of antigen.

Preparation of the Antigen. — Suckling mice were inoculated at the age of 4—6 days by the intracerebral route using a 10⁻⁵ dilution of infectious suckling mouse brains containing about 1,000 LD₅₀. On the fifth day after the inoculation the mice were anesthetized with ether and exsanguinated by cutting open the thorax. The brains were harvested and crushed with quartz sand in a mortar. A 10 per cent suspension was made with 0.05 M borate—0.12 M NaCl, pH 9.3. The alkaline brain suspension was immediately centrifuged at 11,000 g for one hour. The supernatant fluid constituted a stock antigen, which was stored at +4°C.

Hemagglutination Test. — The antigen was diluted with a 0.05 M borate—0.12 M NaCl—0.4 per cent bovine albumin³ solution, pH 9.0, in twofold dilutions beginning with 1: 100. The volume of antigen dilutions in tubes was 0.5 ml. The tubes were chilled in a refrigerator at +4°C before addition of erythrocytes.

The erythrocytes were taken from a wing vein of the fowls into syringe containing a sodium citrate—citric acid—dextrose solution as anticoagulant. After filtration through a sterile gauze the erythrocytes were washed four times with saline. A 0.25 per cent erythrocyte suspension was made volumetrically into 0.20 M phosphate—0.15 M NaCl buffers, the proportion of monobasic and dibasic sodium phosphate being adjusted to yield final pH values from 6.0 to 7.0 after mixing with equal volumes of borate-saline-albumin, pH 9.0.

²The author thanks Mr. O. Kallela and Mr. I. Palva for kindly permitting him to take blood samples from their poultry.

³Bovine plasma albumin, fraction V of the Armour Laboratories

All the samples of erythrocytes were examined at pH 6.0, 6.2, 6.4, 6.6, 6.8, and 7.0. The settling of erythrocytes took place usually at +4°C and a part of the samples were studied also at +20°C and +37°C. The results were read only after the complete sedimentation of erythrocytes. The preparation of chemical reagents and other technical details have been recently published by Clarke and Casals (6), to which reference is made.

RESULTS

The agglutinability of the individual erythrocyte samples from the different groups of fowl at the optimum pH and temperature is presented in Table 1. It shows that all the samples of erythrocytes from adult roosters were agglutinable by the hemagglutinin to an average titer of 1: 12,800. On the other hand, two-thirds of the erythrocyte samples from adult hens were completely negative, whereas one-third of the hen erythrocytes showed variable titers from 1: 1,600 to 1: 12,800. All the samples of one-day-old chick erythrocytes reacted positively up to an average titer of 1: 25,600. Approximately in the same manner reacted also the adult goose erythrocytes.

TABLE 1
AGGLUTINABILITY OF ROOSTER, HEN, CHICK AND GOOSE ERYTHROCYTES BY TICK-BORNE ENCEPHALITIS VIRUS HEMAGGLUTININ AT THE OPTIMUM pH 6.4 OR 6.6 AT +4°C

Group of Fowl	Age	Number of Individuals Studied	Positive per cent	Agglutination Endpoints of Erythrocytes in Hemagglutinin Dilutions						
				> 1: 100	1: 1,600	1: 3,200	1: 6,400	1: 12,800	1: 25,600	1: 51,200
Rooster	1—4 years	26	100 %	—	—	—	—	23	3	—
Hen	1—4 years	24	33 %	16	3	2	2	1	—	—
Chick	1-day-old	20	100 %	—	—	—	—	1	18	1
Goose	1—2 years	2	100 %	—	—	—	—	1	1	—

The agglutination zones of one sample each of rooster, chick and goose erythrocytes at different pH's and temperatures are shown in Figure 1. The agglutination zones of other samples of erythrocytes so far studied were very similar to those presented in this figure. The following conclusions may be drawn from these studies: The optimum pH for agglutination was 6.4 or 6.6. The highest hemag-

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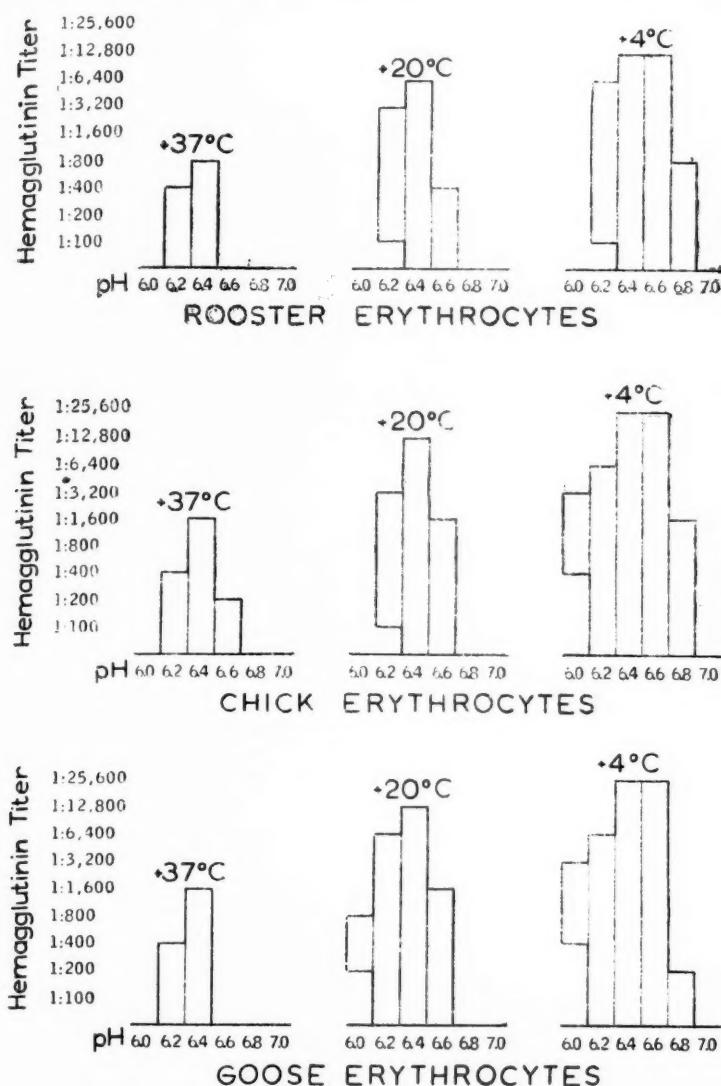


Fig. 1. — Agglutination Zones of Rooster, Chick and Goose Erythrocytes at Different pH's and Temperatures

glutinin titer was always obtained at +4°C. The titer was one-half as high at room temperature and 1/16 as high in a water-bath at +37°C as at the refrigerator temperature. With increasing temperature the pH range of agglutination became narrower. In the acid part of the agglutination zone a prozone tended to occur.

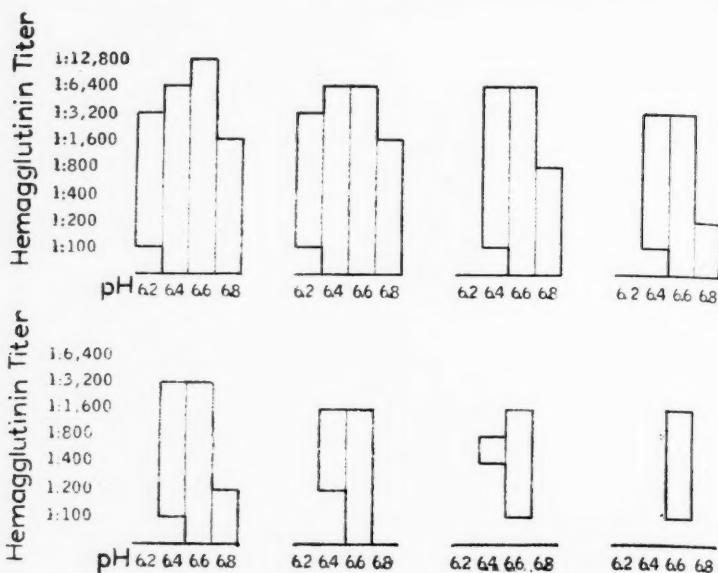


Fig. 2. — Agglutination Zones of the Reacting Samples of Hen Erythrocytes at Different pH's at +4°C

The agglutination zones of the positively reacting samples of hen erythrocytes differed from the above with a few exceptions. As seen in Figure 2, the pH range of agglutination became narrower at the same time as the agglutination endpoint of erythrocytes became lower. Incomplete settling patterns of erythrocytes were also more often seen with adult hen erythrocytes. The reactivity of hen erythrocytes was not correlated to the age of a hen within the limits of 1—4 years.

The addition of protein to test solutions is necessary for goose erythrocytes because they tend to agglutinate spontaneously in pure solutions of salts. Protein is not necessary, however, when rooster, hen or chick erythrocytes are used. For the sake of uniformity all the experiments reported here were performed in 0.2 per cent albumin solutions.

Since the discovery of the hemagglutinating property of rooster erythrocytes by the tick-borne encephalitis virus, rooster erythrocytes have been used routinely for the hemagglutination inhibition studies of serums. The experience from the examination of about 500 serums proved the suitability of rooster erythrocytes for

hemagglutination inhibition studies. The results were identical with those obtained with one-day-old chick or goose erythrocytes. Different samples of rooster erythrocytes were equally sensitive to the hemagglutinin and we never found rooster erythrocytes that did not possess agglutinating capacity.

DISCUSSION

The results reported above showed that adult rooster erythrocytes were without exception agglutinable by tick-borne encephalitis virus hemagglutinin to a constant high titer, while the majority of hen erythrocytes were negative. A third of the hen erythrocytes, however, showed various degrees of agglutination and among them a gradual shift of agglutinability from positive to negative was observed.

Since no difference could be found in the reactivity of erythrocytes from newly hatched chicks, it is evident that the majority of hens later lose the agglutinability of their erythrocytes. This would for instance occur by inactivation or release of the virus receptors on the surface of erythrocytes. The mechanism of the phenomenon is not yet known, but the change may be caused by female hormones.

If it can be demonstrated that rooster erythrocytes are agglutinable also by the other members of the arthropod-borne group, rooster erythrocytes may be recommended for routine use in hemagglutination inhibition tests with the arbor viruses.

SUMMARY

A sex-linked difference was found in the agglutinability of rooster and hen erythrocytes when a strain of the Russian biphasic tick-borne encephalitis virus was used as antigen.

All the samples of erythrocytes from adult roosters were agglutinable by the hemagglutinin of the tick-borne encephalitis virus to a constant high titer. The agglutination zones of rooster erythrocytes at different pH's and temperatures were comparable to the results obtained with one-day-old chick or adult goose erythrocytes.

On the contrary, two-thirds of the erythrocyte samples from adult hens were not at all agglutinated by the hemagglutinin.

Among one-third, a gradual shift from positive to negative was observed in the agglutinability. The reactivity of hen erythrocytes was not correlated to the age of the fowl. No difference was seen in the agglutinability of erythrocytes from newly hatched chicks.

Rooster erythrocytes were found to be suitable for studies of hemagglutination and hemagglutination inhibition by the tick-borne encephalitis virus.

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THE CHOLINESTERASE ACTIVITY OF THE UTERUS AND FRANKENHÄUSER'S GANGLION

A HISTOCHEMICAL STUDY

by

S. MÄKELÄ and M. GRÖNROOS

(Received for publication June 22, 1959)

The contractions of the uterus and the hormonal and neurogenic factors effecting them have always been subject to a most vigorous study as well as a great deal of controversy in obstetrics. As early as 1867 Frankenhäuser (4) studied the problem from the histo-neurologic point of view and was the first to describe the increase of the cervical plexus during pregnancy. Calman (2), in 1898, supposed the occurrence of changes in sensibility during pregnancy. This supposition was supported by the studies of Hansen (8). According to him the nerve fibres of the genitals become thicker and increase in number during pregnancy.

With the improvement of the histochemical methods of investigation more and more light has been thrown on these problems. E.g. Gasparini (6) has observed hypertrophy and changes in the nucleus-plasma index in the neurons of the cervical ganglion during pregnancy both in man and in cattle. He attributed the changes to the enlargement of the innervated area. Gabrielescu and Bordeianu (5) noticed that a prolonged presence of stimuli (estrus and gravidity) effect an increase in the number of terminal nerve fibres in the uterus of rabbit. Using the thiocholine technique Koelle (10) demonstrated cholinergic peripheral nerve fibres of cat, rabbit and monkey in 1955. In 1956 Gastaldi (3) published his study on

the uterus on woman, where he pointed out the existence of a great number of cholinergic fibres particularly in the cervix uteri.

Since the writers have not come across any papers dealing with the changes in the cholinesterase activity of the cholinergic fibres of the uterus and with those in the neurons of the Frankenhäuser's ganglion under different physiological conditions, and since these questions might be of importance with respect to the dynamics of the uterus, it has been the intention of the writers to throw light upon the question.

MATERIAL AND METHOD

The series consisted of 30 female albino rats weighing about 175—200 g. The rats were distributed into five groups:

I Group: rats in diestrus (the having been determined according to Shorr's staining method; Smolko and Soost [13]).

II Group: gravid rats; the duration of gravidity from 5 to 7 days.

III Group: gravid rats; the duration of gravidity about 20 days.

IV Group: rats with litter 4—5 days previously.

V Group: rats having undergone oophorectomy 5 months previously.

The test animals were killed by hitting them on the head. A solution of 10% neutral formaline was immediately injected into the left ventricle. The uterus was quickly excised and placed into cold (4°C) 10% neutral formol. After fixation (2 hours) the uteri were cut with a freezing microtome and the slips were treated according to Gomori's (7) modification of Koelle's method. The incubation time was $\frac{1}{2}$ —2 hours.

In the examination of the specimens the attention was centered on the cholinesterase activity of the cholinergic fibres of the myometrium and on that of the neurons in the Frankenhäuser's ganglion.

RESULTS

Uterus. — In agreement with Castaldi's findings with respect to the human uterus, the writers observed abundant cholinesterase activity, which occurred mainly as granulation in the fibrous structures. The fibres were observed to be anastomosing with each other to a great extent, forming net- and braid-like structures (Fig. 1 and 2), which, again, were often situated round blood vessels. This was the case in the area of corpus and particularly in that of the portio. In the cornua of the uterus (Fig. 3) the activity was mainly observed in the longitudinal layer of myometrium. The

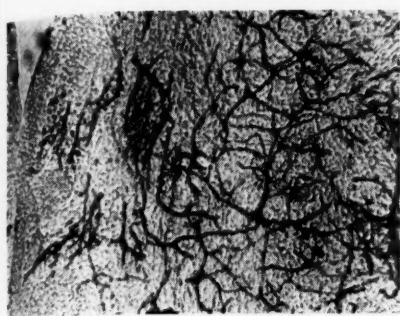


Fig. 1.—The activity of the non-specific ChE in corpus uteri near the portio. Diestrus rat. $\times 220$.

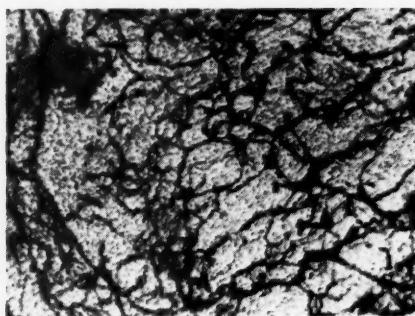


Fig. 2.—The activity of the non-specific ChE in corpus uteri near the portio. Oophorectomized rat. $\times 220$.

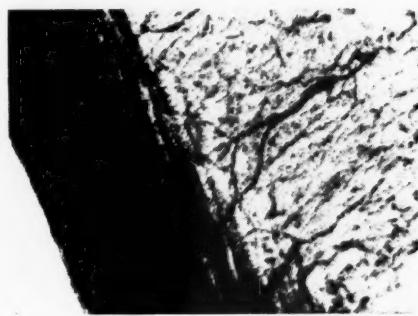


Fig. 3.—The activity of the non-specific ChE in cornu uteri. Destrus rat. $\times 220$.

activity seemed to be due to non-specific cholinesterase in the first place.

Frankenhäuser's Ganglion. — In the cells of the ganglion there was already observed abundant granulation after an incubation period of half an hour (Fig. 4 and 5). Still, the cholinesterase activity differed *e.g.* from that observed in ganglion nodosum (Fig. 6 and 7) both as regards its intensity and its locality. In ganglion nodosum the specific cholinesterase was present almost exclusively in the cytoplasm of the neurons and the non-specific cholinesterase outside the neurons, *e.g.* in the satellite cells. In the Frankenhäuser's ganglion it was somewhat more difficult to specify the locality of these enzymes. When incubating with AThChX¹ it was possible to observe, however, that granulation mostly became localized

¹ (Acetylthiocholine)

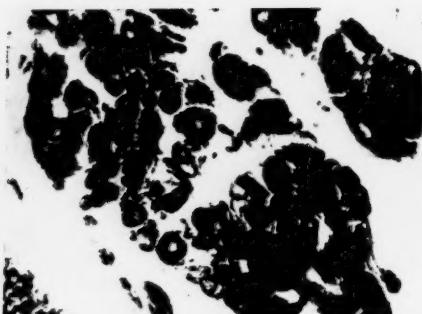


Fig. 4. — The activity of the AChE in the Frankenhäuser's ganglion. Diestrus rat. $\times 220$.

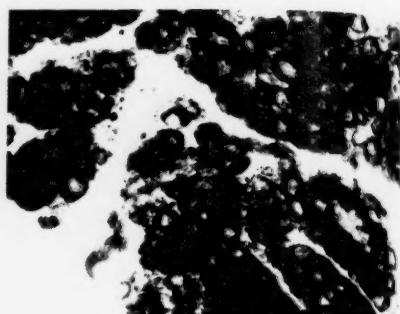


Fig. 5. — The activity of the non-specific ChE in the Frankenhäuser's ganglion, Diestrus rat. $\times 220$.

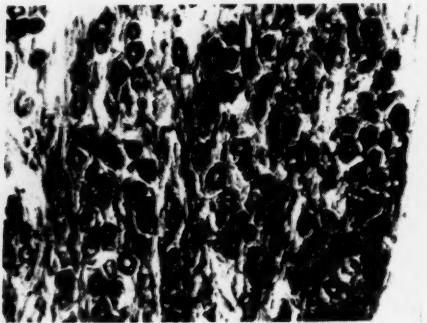


Fig. 6. — The activity of the AChE in the ganglion nodosum. Diestrus rat. $\times 220$.

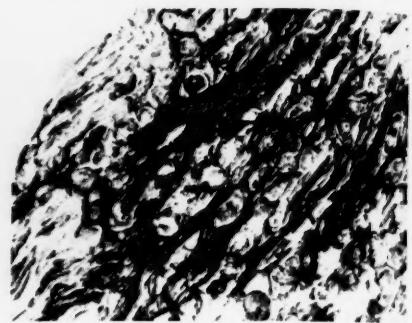


Fig. 7. — The activity of the non-specific ChE in the ganglion nodosum. Diestrus rat. $\times 220$.

in the periphery of the cells. The greatest concentration of the granulation was centered on the satellite cells when the incubation was effected by BuThCh.¹ The existence of non-specific cholinesterase was to be observed in the cytoplasma of the neurons in all the specimens to some extent; still, the part played by a diffusion artefact has also to be taken into consideration. On the basis of acetylcholine activity there could be observed three kinds of neurons in one and the same ganglion: strong, moderate and weak or no activity. The extent of the non-specific cholinesterase was so much alike in the different groups that no clear classification can be attempted.

¹ (Butyrylthiocholine)

COMPARISON OF THE GROUPS

I Group acted as a control series of a kind. What has been said above holds good with respect to it.

II Group did not differ clearly from the I group.

III Group mostly differed from the I group in the fact that there were more fibres in the uterus. Many of the fibres were spiral-like and thicker than those in the two first groups. However, the activity seemed to be somewhat weaker than in the preceding groups. In the Frankenhäuser's ganglion the activity was also slightly weaker on the average than in the »diestrus series».

IV Group: the ChE activity of the uterus was approximately of the same extent as that in the I group. In the Frankenhäuser's ganglion the neurons exhibited strong AChE activity, which was often even more vigorous than that in the I series.

V Group differed most clearly from the other groups. The cholinergic fibres were thick and very dense in the uterus near the portio (Fig. 2). Enzyme activity was of the same extent as that in the »diestrus series». The cornua of the uterus had shrunk thin like thread. The cholinergic fibrosity of the longitudinal layer of myometrium was there of a smaller density. In the Frankenhäuser's ganglion the extent of granulation was considerable and corresponded best to the I group in this respect.

DISCUSSION

Due to the staining method used in the present investigation no exact and detailed determinations of the cell structures can be made; most obvious difficulties are also due to diffusion artefacts. Still, as has been pointed out by Koelle (10), the thiocholine technique can be made use of in the identification of cholinergic neurons as well as in comparing the nerve fibres with respect to their individual content of cholinesterase. It is also to be admitted that the study is rendered more complicated by the fact that the differences between various esterases are only relative and not absolute; they vary from one species to another, frequently even from one animal to another.

According to Uranga Imaz *et al.* (14) the cholinesterase content of the blood does not vary during pregnancy or puerperium. It is

obvious, however, that no definite conclusions can be drawn on the basis of the blood with respect to the occurrence of enzymes in the uterus (Burger 1.). According to the present observations the number of cholinergic fibres was increased towards the termination of gravidity, whereas the cholinesterase activity of individual fibres was of a smaller degree than that in the »diestrus series», *i.e.* the relative cholinesterase content of the uterus had decreased. Because of this it could be supposed that the predisposition to contraction would have been decreased since a lower cholinesterase content in other organs denotes »unempfindlichkeit auf acetylcholin» (Sawyer *et al.*, 12). An interesting theory by Burger (1) is worth mentioning in this connection. According to him the uterine acetylcholine-cholinesterase-system mainly regulates the function of the blood vessels, and the sexual hormones, the contents of which undergo changes parallelly with the cholinesterase of the endometrium, have an activating influence on this system and on other biochemical processes as well.

Another observation of importance is the fact that the cholinergic fibres were of a considerable density in the shrunken uteri of the rats in the V group (comprising rats that had undergone oophorectomy). This finding is somewhat contradictory to those published by Burger; yet it has to be borne in mind that the latter only dealt with the endometrium. On the basis of the present observation it would seem adequate to suppose that the ChE-activity of the cholinergic fibres of the myometrium does not undergo a permanent change although the ovaries are removed and the hormonal balance is thus greatly disturbed.

Finally it is worth pointing out that the literature on the subject contains exceptionally much contradictory information about the autonomic innervation of the uterus as well as about the nerve fibres of the myometrium. One explanation of this unusual inconsistency of opinion is offered by Jabonero (9), who supposes a common »end-organ», syncytium, in the uterus acting both for the sympathetic and the parasympathetic. This end-organ, which consists of protoplasmatic nerve fibres, should send the centrifugal impulses, which it receives from the autonomic fibres, to the peripheric cells. This syncytium might be adrenergic. However, the question still remains open for further studies. It may only be pointed out that effects of adrenaline and acetylcholine on the

uterus vary in different animal species (Loewe 11). The writers are working on a study whose purpose it is to find out the relation between the observed cholinergic fibres and those protoplasmatic nerve fibres described by Jabonero.

SUMMARY

By using Gomori's modification of Koelle's thiocholine method the writers have studied the cholinesterase activity of the cholinergic fibres of the uterus and that of the neurons in the Frankenhäuser's ganglion in rats. It was observed that gravid rats exhibited more cholinergic fibres in the uterus compared with the rats in the diestrus series, but that the enzyme-activity was lower in the former group. It was further observed that a bilateral oophorectomy did not bring about any changes in the number of cholinergic fibres or in the enzyme-activity. The significance of the observations is discussed.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY
UNIVERSITY OF HELSINKI

FAILURE TO DEMONSTRATE AN ACQUIRED
IMMUNOLOGICAL TOLERANCE OF CHILDREN TO
THE ABO-AGGLUTINOGENS OF THEIR MOTHER

by

ANJA TIILIKAINEN¹, RAIMO LEHTOVAARA, and A. W. ERIKSSON

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Only a small part of the Rh-negative women who have Rh-positive children produces Rh-antibodies. The conception of immunological tolerance has given rise to the question whether the Rh-negative daughters of Rh-negative women would more often be immunized by their Rh-positive children than the Rh-negative daughters of Rh-positive women. This rather interesting theme has been studied by various authors, but the conclusions are not exactly unanimous (1, 2, 3, 4). In the present investigation the effect of mother's ABO blood group on the isoagglutinin titers in children has been studied in order to see whether these antigens might induce a tolerance phenomenon.

MATERIAL AND METHODS

159 sera of A, B, and O persons aged 1 to 40 years have been studied. Their mother's ABO blood groups were known. All sera were reserved in -20° C and all tests were carried out simultaneously.

The test cells of type A₁ and B were washed three times with physiol. saline solution, and for use $\frac{1}{2}\%$ suspensions in saline and undiluted AB-serum were made.

¹ Aided by a grant from the University of Helsinki.

FIG. 1.

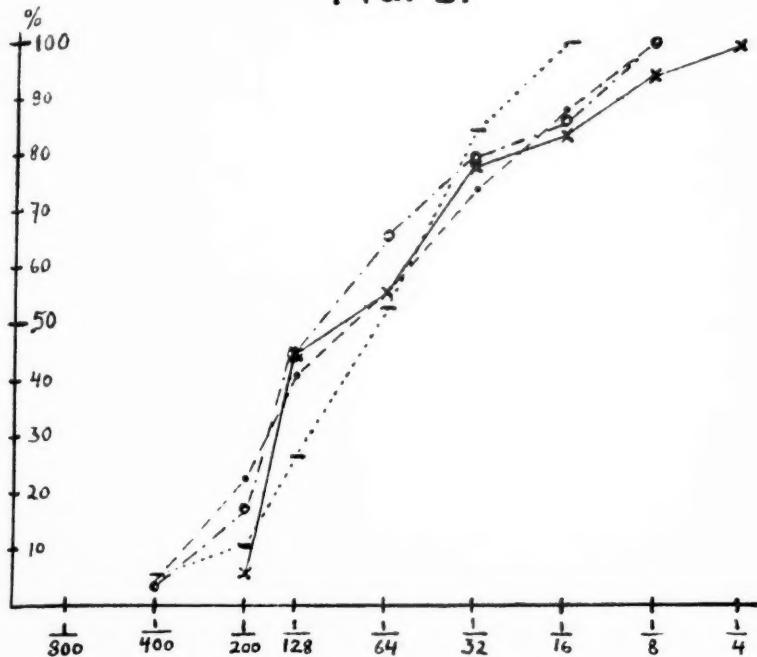


Fig. 1. — Anti-A titres in children

Blood group of child	A -substance in mother	N: no of cases
x—x	B present	18
o---o	B not present	29
-·-·-	O present	19
-·-·-	O not present	27

Twofold dilutions of the sera were made in saline in bulk, and one volume of each dilution was put in a series of round bottomed test tubes. Then one volume of testcells in saline was added and the tubes were incubated in +20° C to probe saline isoagglutinins. 100 of the sera were simultaneously tested for »incomplete» isoagglutinins. For that reason another series of equal volumes of twofold dilutions and cells in AB-serum was incubated in +37° C. All the reactions were read after 3 hours.

RESULTS

In figure 1 the saline anti-A titres of »tolerant» and »nontolerant» persons have been compared. In figure 2 the corresponding compari-

FIG. 2.

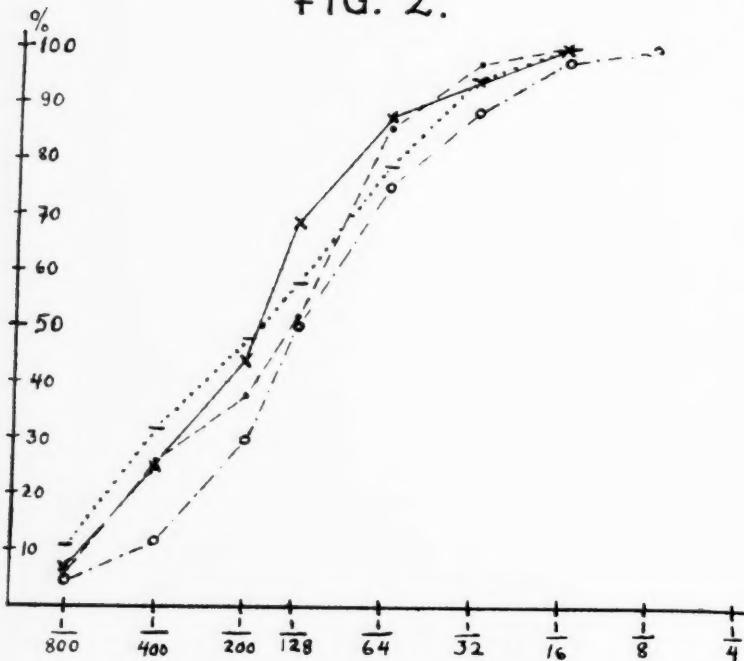


Fig. 2. — Anti-B titres in children

Blood group of child	B -substance in mother	N: no of cases
x—x	A present	16
o---o	A not present	44
- - - -	O present	11
- - - -	O not present	35

son of anti-B titres has been made. There are no differences between these two groups of persons. Similar results were obtained when the sera were titrated in AB-serum.

In figures 3—4 differences in the titres between anti-A and anti-B of the individual O-sera are calculated. The number of these O-sera tested was 52.

No significant differences can be seen in the results.

DISCUSSION

Only few of our mostly young test persons might have been really immunized. Blood transfusions, pregnancies, diphtheria and

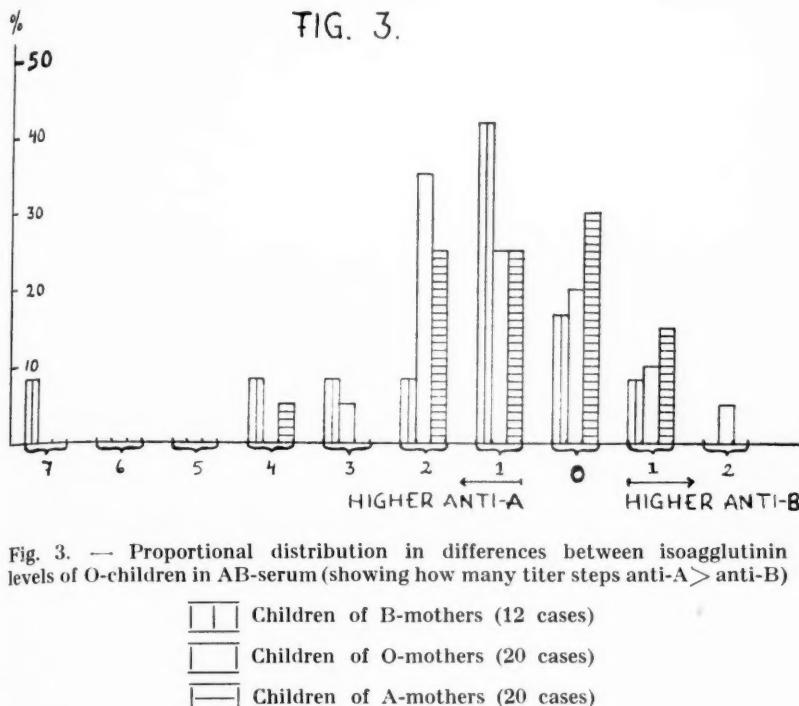


Fig. 3. — Proportional distribution in differences between isoagglutinin levels of O-children in AB-serum (showing how many titer steps anti-A > anti-B)

tetanus toxoids and tetanus antitoxin injections may appear as such immunizing agents. Accordingly, «immune» isoagglutinins in healthy persons obviously are not on their maximal level, which condition would be most favourable for studies of the tolerance phenomenon. The immunization of volunteers probably would give most reliable results.

In figure 3 the departures agree with the hypothesis though they are far from significant.

The immunological tolerance in anti-A levels is tested only with A_1 cells. Consequently the effect of A subgroups is not to be seen in our experiments, and it seems that the possible tolerance phenomenon would reveal itself better in anti-B levels than in anti-A levels.

Altogether, according to our investigations it is likely that if the human isoagglutinin level is influenced by the blood group of mother, it is rather difficult to demonstrate.

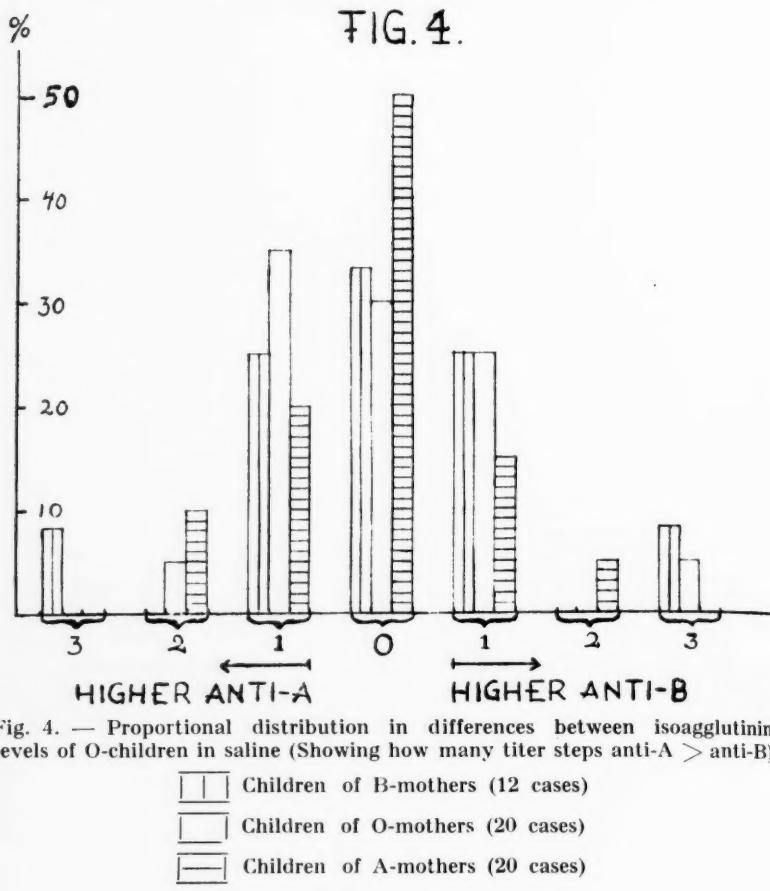


Fig. 4. — Proportional distribution in differences between isoagglutinin levels of O-children in saline (Showing how many titer steps anti-A > anti-B)

[] Children of B-mothers (12 cases)

[] Children of O-mothers (20 cases)

[] Children of A-mothers (20 cases)

SUMMARY

Isoagglutinin levels of 159 Finnish persons aged 1 to 40 years have been tested in order to see whether the ABO blood group of mother is reflected in lower titres of respective antibodies in children. No significant effect was found.

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FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF TURKU

EFFECT OF FLAVASPIDIC ACID AND MALE FERN EXTRACT ON GRAVIDITY

AN EXPERIMENTAL STUDY WITH ALBINO RATS

by

T. K. MARKKANEN, M. GRÖNROOS, and T. VANHA-PERTTULA

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The occurrence of circulatory disturbances has been observed clinically in connection with vermicides. Experimentally the flavaspidic acid and male fern extract have a clear effect on the heart muscle in vitro (8 and 9). They have also been noticed to command a toxic effect on the circulatory system in vivo (4). Clinical experience indicates that these substances would also have untoward effects on the uterus. It has been possible to demonstrate in vitro that flavaspidic acid and male fern extract effect the uterus either as a relaxant or as a tonic depending on the contemporary state of the muscle at the moment. The following is an experimental study on the effects of these vermicides on the gravidity of rats.

METHOD

The series consisted of 46 female albino rats at the age of four months and weighing 160—260 g. The animals had not been gravid before. At the beginning of the experiment the rats were weighed and placed into cages in groups of six with one male for ten days. The beginning of gravidity was ascertained by observing the vaginal smear. After the copulation period the gravid animals were grouped into six series. The substances were given per os and only one time. Flavaspidic acid was suspended in water and the male fern extract

in olive oil. Table 1 shows the number of animals in each group, the substance and the dose per one gram of body weight.

After the administration of the vermicifuges the animals were placed in single cages for observation. The animals were weighed every second day. Vaginal smear samples were taken of all the animals every third day. The animals that had not littered or undergone abortion within 34 days were killed and obducted. Both macro- and microscopical examinations of the uteri were carried out. The specimens were stained according to van Gieson's method.

RESULTS

The number of normal litters and abortions observed in each group are given in Table 1. More detailed information about each group is given in the following.

TABLE 1

Group	Number of rats	Drug	Dose mg/g	Normal Litter	Abortion
I	9	Flav. acid.	0.02	4 (44 %)	5 (56 %)
II	8	—	0.77	1 (12 %)	7 (88 %)
III	6	—	1.38	2 (33 %)	4 (67 %)
IV	10	Male fern extract	0.54	5 (50 %)	5 (50 %)
V	5	—	1.11	0	5 (100 %)
VI	8	5 cc H ₂ O or Ol. oliv.		8 (100 %)	0

I Group (Flav. acid. 0.02 mg/g):

Four animals (44%) littered normally within the 23th and the 27th day of the experiment. The offspring appeared normal and weighed 4.5—6.5 g. Two rats littered between the 23rd and the 26th day, one with ten offspring, weighing 1.9—2.5 g. All the offspring were born dead. The remaining three rats of the series exhibited remnants of gravidity in the uteri.

II Group (Flav. acid. 0.77 mg/g):

One animal had a normal litter on the 28th day of the experiment. Three animals littered prematurely on the 18th day. The offspring alive and weighed, on the average, about half of the weight of the offspring in the control series. At obduction, remnants of gravidity were found in the uteri of the remaining four rats.

III Group (Flav. acid. 1.38 mg/g):

Two animals had a normal litter on the 26th day of the experiment. Dead macerated abortive fetuses were born by one animal on the 18th day. At the obdunction remnants of gravidity were found in the uteri of the remaining three rats.

IV Group (Male fern extr. 0.54 mg/g):

Five animals (50%) had normal litters between the 24th and 28th day of the experiment. On the 32nd day one animal bore a dead macerated offspring which looked like an underdeveloped fetus. Blood was observed in the vaginal smear samples of the remaining four animals even at the beginning of the experiment. Obdunction revealed remnants of gravidity in the uteri of these rats.

V Group (Male fern extr. 1.11 mg/g):

None of the animals in this group littered normally. Between the 12th and the 18th day of the experiment two animals had an abortive litter: living premature fetuses, weighing about half of the normal weight, by one and a badly macerated dead fetus by the other. At obdunction both macro- and microscopical examinations revealed signs of abortive gravidity in three animals.

VI Group (control group):

All the animals in the control series had normal litters, the offspring weighing 4.5—6.5 g, between the 22nd and the 30th day of the experiment. Weighings, carried out every two days, revealed that the average increase in weight was greater than that in any of the preceding groups. A slight decrease in weight took place even in this group during the couple of days following the administration of the dose. In all the test series the decrease was greater than in the control series. The decrease was the most obvious in the II group, which also exhibited the slightest increase in weight during the experiment.

Considering all the animals that were given greater or smaller doses of vermifuges, it will be observed that the normal course of gravidity was disturbed in almost 70 per cent of the cases. Blood was also detected in the vagina in most of these cases. The animals did not exhibit signs of poisoning to any noteworthy degree. Only a few showed a loss of appetite and other slight symptoms of illness.

DISCUSSION

Flavaspidic acid and male fern extract are the most common drugs used in Finland as vermifuges for broad tapeworm. Still,

the occurrence of some circulatory disturbances has been observed clinically in connection with them (3,7). Electrocardiographic changes, dyspnea, vertigo and even signs of shock have been noted. In slight cases the disturbances have disappeared a few days after the termination of the vermifuges. Some occurrences of death have also been met with in this connection. The patients suffering from complications in connection with vermifuge have been over 40 years of age as a rule and they have also exhibited coronary sclerosis and signs of myocardial disorders. So far there exists no conclusive evidence on the question whether the disturbances are brought about by vermifuges proper, or by laxatives, or by those two in combination. It is to be supposed that a drastic evacuation of the bowels by means of magnesium sulfate or by enema should have noxious effects on a heart affected by coronary disorders.

In animal experiments it has also been possible to demonstrate that flavaspidic acid and male fern extract have noxious effects on the heart muscle both *in vivo* and *in vitro* (4, 8, 9). When injected into living test animals they produce a considerable rise in the blood pressure within the pulmonary circulation even in rather small doses (4). They have also been observed to have an inhibitory effect on certain enzymatic processes regulating the functioning of the muscles both in the musculature of the heart and the uterus.

The effect of flavaspidic acid and male fern extract on the musculature of the uterus is also of importance for practical purposes. Under *in vitro* conditions the effect of the drugs is somewhat variable depending on the state the uterus of the animal has been in; *e.g.* on a uterus in the estrus phase they act as relaxants whereas their effect is tonic on a gravid estrus (10). There has already been a tendency to avoid the giving of vermifuges to pregnant patients partly because of the fact that laxatives in connection with vermifuges probably lead to abortions, partly because the possible disturbances brought about by teniafuges on the uterus are not known. Neither is the resorption of these substances from the bowels known, although they form alkaline salts that are soluble in water.

The results of the present animal experiments indicate that flavaspidic acid and male fern extract would have a clearly disturbing effect on the course of gravidity. This is clearly obvious even when small concentrations of flavaspidic acid are made use of. The mechanism of the effect produced by these drugs on a gravid

uterus *in situ* cannot be determined with certainty. Still, the relatively large number of animals that exhibited remnants of gravidity deserves special attention. Whether flavaspidic acid and male fern extract differ from each other in their effects on gravidity cannot be answered on the basis of the present experiments. In the I group, where the flavaspidic acid dose was near a single human vermifuge dosage, the number of affected gravidities was about a half of the total number of animals in the series. Yet doses that were several times stronger than the first one did not increase the number of abortions in any considerable degree. It is impossible to infer whether a vermifuge administered during the early stage of pregnancy would have a disturbing effect on the development of the embryo in case the pregnancy should not end in an abortion.

SUMMARY

The effect of different concentrations of flavaspidic acid and male fern extract on the gravidity of rats was studied. The drugs were administered per os. The substances were observed to have an abortive effect. The question whether the effects produced by flavaspidic acid and male fern extract respectively differ from each other cannot be answered on the basis of the present experiment.

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FROM THE MEDICAL DEPARTMENT, MARIA HOSPITAL AND THE FOURTH
DEPARTMENT OF MEDICINE, UNIVERSITY OF HELSINKI

STUDIES WITH I^{131} -LABELLED FAT¹

I

THE ESTIMATION OF INTESTINAL FAT ABSORPTION USING I^{131} -LABELLED OLIVE OIL

by

M. SIURALA, B.-A. LAMBERG, K. TURULA, and P. HÄRKÖNEN

(Received for publication August 4, 1959)

Fat labelled with radioactive iodine has been used recently for the detection of abnormalities in the intestinal absorption of fat. The method has been found valuable in the diagnosis of chronic pancreatic disease and of other disorders characterized by defects in fat absorption (1—8, 10—23, 25).

In order to overcome the practical difficulties associated with fecal radioactivity determinations various modifications of the blood radioactivity measurements originally introduced by Stanley and Thannhauser (26, 27) have been extensively used. Fat absorption has been evaluated on the basis of circulating radioactivity in whole blood, plasma or the precipitate obtained after trichloroacetic acid precipitation (1—8, 11—16, 18—24). The blood radioactivity at a certain time after a labelled fat test meal is obviously only a snap-shot of a most complicated process of distribution which will be discussed in a subsequent paper. The present authors suggested in a preliminary report, therefore, that trials should be made to calculate the total amount of absorbed

¹ This study was aided by a grant from the Sigrid Jusélius Foundation.

radioactivity at a given time following a test meal of radioactive olive oil (9).

This paper is a report on our short-term experiments to study the radioactivity in whole blood and trichloracetic acid precipitate of whole blood in normal and pathological conditions of the gastrointestinal tract.

MATERIAL AND METHODS

Thirty-six pathological cases were studied. Five patients suffered from chronic relapsing pancreatitis, one had chronic pancreatitis and had previously undergone partial resection of the pancreas for cyst-formation in the gland, three had idiopathic steatorrhoea and the remaining 27 cases had had an attack of acute pancreatitis one to five years prior to this investigation. The control cases were 21 patients treated for various diseases but free of clinical or roentgenological symptoms of a gastrointestinal disorder.

Pure olive oil was iodinated directly with radioactive elementary iodine I^{131} (Amersham, England). The iodination procedure was carried out at room temperature during 2 days. The iodinated olive oil was washed once in alkaline sodium thiosulfate solution and thereafter twice with distilled water to remove the inorganic iodide. Between 20 to 50 μ C of the radioactive olive oil was administered after a 12-hour overnight fast as a test meal containing 15 ml of olive oil, approximately 30 g of white bread and 200 ml of carbonated beverage. In the early experiments the labelled olive oil was pipetted into the white bread, but later on it was administered separately in a gelatine capsule.

Thyroid uptake of radioactive iodide was prevented by giving the patients 250 mg of potassium iodide three times daily on the day before and during the days of the test.

In the pathological cases, blood was drawn 4, 5 and 6 hours after the test meal. In the control cases blood samples were taken 2, 4, 6, 8, 10 and 24 hours after the meal and the urine was collected parallelly. The present report, however, is concerned only with the 2, 4, 6 and 8 hour blood determinations and the 6 hour urinary excretion. The blood was drawn in oxalate. The radioactivity in 4 ml of whole blood was measured. Following this, precipitation was carried out according to Beres, Wenger and Kirsner (3) with 20 per cent trichloracetic acid (TCA) and the precipitate activity was measured after two washings in 5 per cent TCA. Before precipitation, 400 mg of carrier iodide was added. The measurements were carried out in a well-type scintillation counter (EKCO type N 550 A) and the values were expressed in per cent of the dose per litre of blood.

Four ml of the urine samples were measured in the same way and the radioactivity in the whole portion was calculated and expressed in per cent of the dose ingested.

An aliquot of the labelled olive oil was diluted in toluene and used as standard solution. Four ml of this solution was counted for activity simultaneously with the blood, precipitate and urine samples.

The fecal radioactivity was measured in two cases with idiopathic steatorrhoea and in 5 control cases. Six grams of charcoal, as suggested by Spencer and Mitchell (25), were given with the test meal and the stools were collected until no trace of the charcoal could be detected. 30 per cent sodium hydroxide was added to the stools, forming a total volume of 1 litre, after which the stools were homogenized. The radioactivity in the whole sample was measured with a flat crystal scintillation counter (EKCO type N 50 B) by placing the container directly on the scintillator head. A standard solution of the same volume, made up in toluene, was measured in the same way. The radioactivity was expressed in per cent of the dose.

RESULTS

The results of the whole blood radioactivity determinations are depicted graphically in Fig. 1. The total radioactivity in the 27 cases which had had an attack of acute pancreatitis before the time of study was wholly within the range of distribution of the controls. Microscopical analysis of the stools revealed, however, considerable amounts of neutral fat in 2 of these cases.

In the three cases with idiopathic steatorrhoea the whole blood radioactivity stayed below the lower limit of the controls. In 2 of the 6 cases with chronic pancreatitis the whole blood values were within the control range.

The results of the determination of the radioactivity in the whole blood precipitate are presented in Fig. 2. In the cases with a previous attack of acute pancreatitis the range of distribution did not significantly differ from that of the controls, although the mean values were slightly lower. On the whole, the precipitate activity showed marked overlapping. In one case with idiopathic steatorrhoea the values were clearly within the control range. The same applied to 2 of the patients with chronic pancreatitis. In addition, 4 other subjects with chronic pancreatitis showed some overlapping with the controls.

Fecal radioactivity in the 5 control cases studied ranged between 4.7 and 6.0 per cent of the dose. In the 2 cases with idiopathic steatorrhoea the fecal excretion values were 11.0 and 22.0 per cent, respectively.

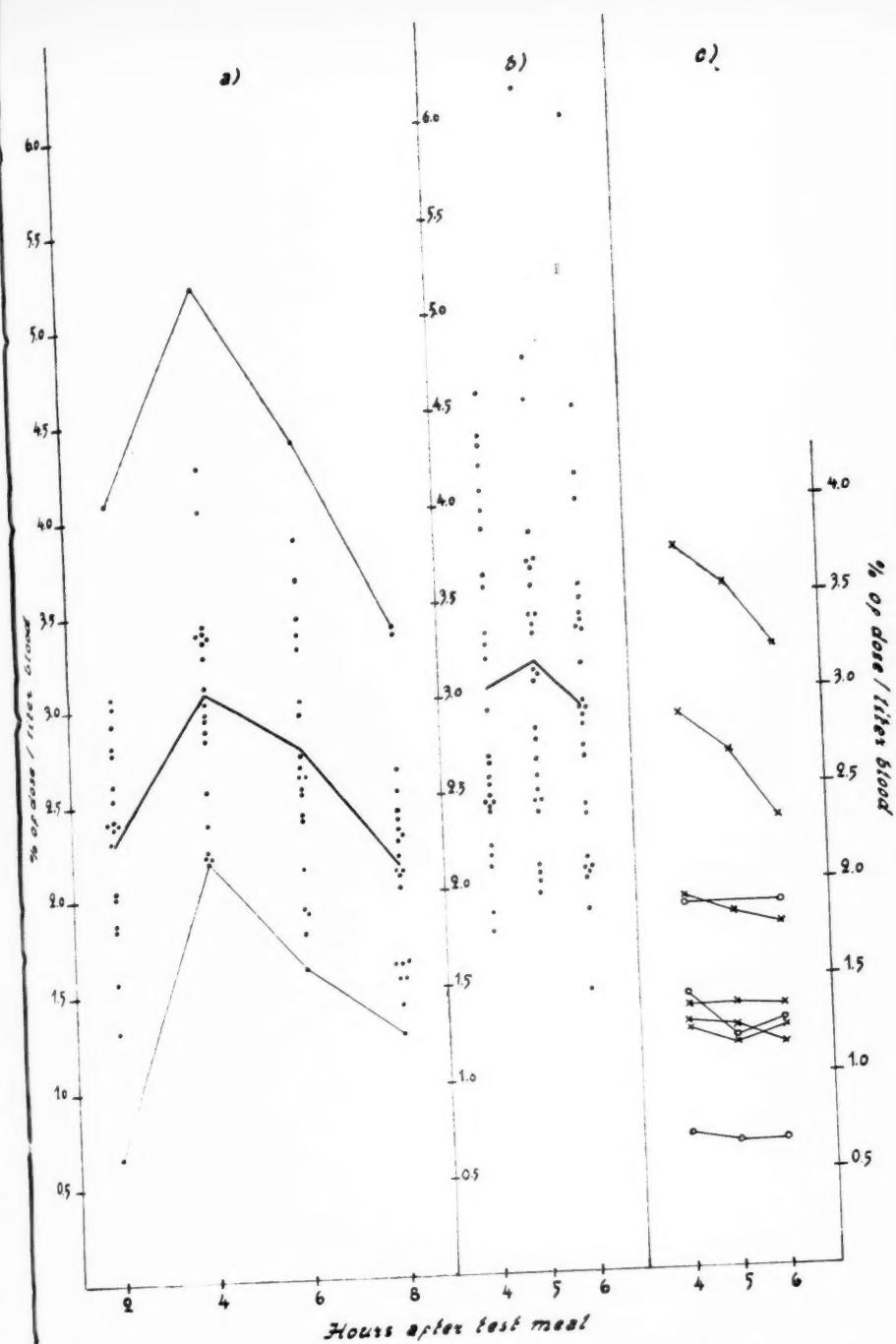


Fig. 1. — Radioactivity in whole blood after ingestion of ^{131}I -labelled olive oil in
 a) 21 control subjects (individual values, range and mean),
 b) 27 patients with one previous attack of pancreatitis (individual values and mean) and
 c) 6 patients with chronic pancreatitis (x ————x) and 3 patients with idiopathic steatorrhoea (o ————o).

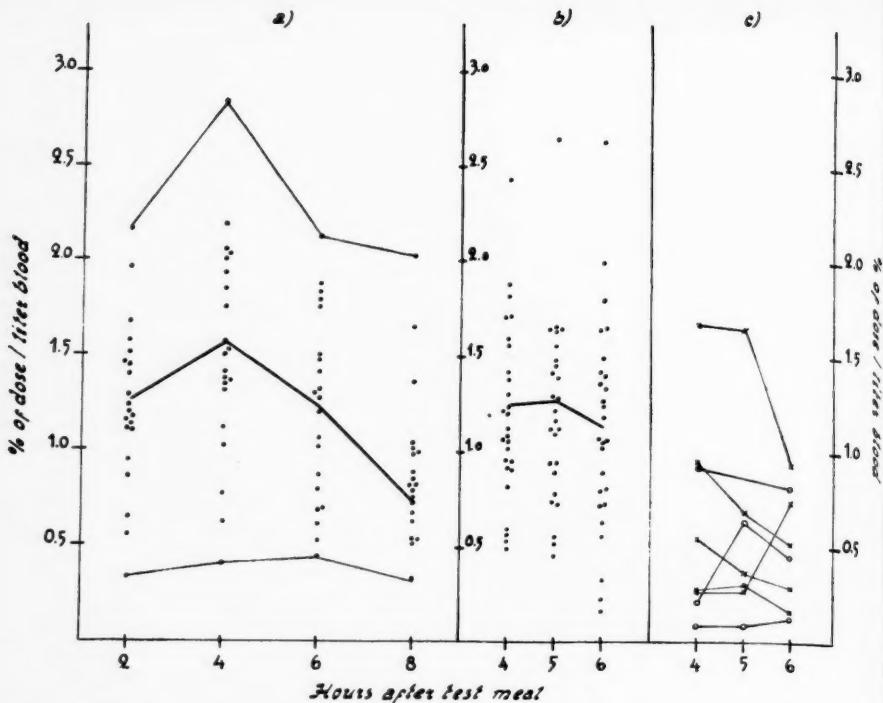


Fig. 2. — Radioactivity of TCA precipitable fraction of whole blood after ingestion of I^{131} -labelled olive oil.
For explanation see legend to Fig. 1.

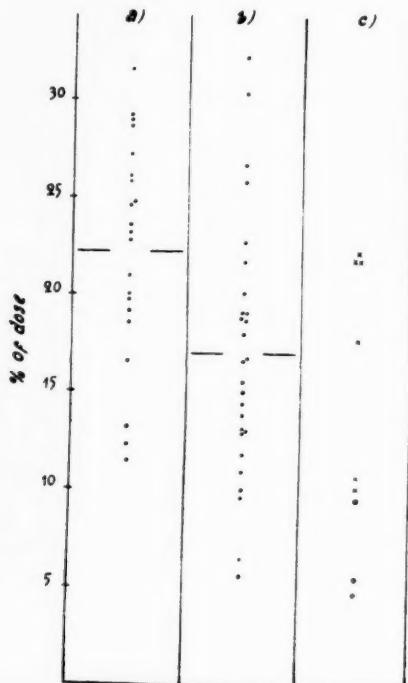


Fig. 3. — Radioactivity in the urine 6 hours after ingestion of I^{131} -labelled olive oil.
For explanation see legend to Fig. 1.

The 6 hour urine activities are presented in Fig. 3. The subjects with chronic pancreatitis showed a tendency toward lower values and were all below the mean of the controls. The 3 cases with idiopathic steatorrhoea were clearly below the lower control limit. As to the cases with one previous attack of pancreatitis, the distribution of urine activity showed a wider scattering in both directions and the mean was somewhat lower than that of the controls.

DISCUSSION

The determination of total blood radioactivity after ingestion of I^{131} -labelled olive oil revealed in all the cases with stetorrhoea values which were below the lower range of the controls. Two out of 6 patients with chronic pancreatitis showed, however, values within the range of distribution of the control subjects. The overlapping was more apparent in the precipitate radioactivity. All cases with a history of one previous attack of acute pancreatitis had values which remained in the main within the range of both whole blood and precipitate values of the controls.

The material is admittedly rather small. The overlapping, however, at least as regards precipitate activity, was fairly evident. Unfortunately the fecal radioactivity was determined in a limited number of cases only and the fecal loss of the 2 patients with chronic pancreatitis and blood values within the control range is not known. The literature reports some overlapping of blood radioactivity between normals and subjects with gastrointestinal absorption disorders, and the correlation between fecal loss and blood radioactivity has not always been satisfactory either (4, 6, 11, 20).

In evaluating further the results obtained from using labelled fat for the detection of disturbances in fat absorption it is evident that there are many sources of potential error. Disturbances in gastrointestinal motility (2, 4, 20, 23) and variations in the composition of the test meal (24, 7) may influence the blood radioactivity. A major point, of course, is the stability of the iodine 131 -fat linkage. Furthermore, metabolic disorders, heart decompensation, arteriosclerosis and certain drugs have been found to affect the results (4, 5, 24, 26, 27).

Many authors have calculated the per cent of the radioactivity dose circulating in whole blood, or the precipitable fraction using

body weight for the estimation of the blood volume (1—5, 7, 11—16, 18—24). The advantages of such a procedure are not apparent. Whole blood radioactivity represents at least two different pools: 1) the iodide diffusion space which is some 4—5 times the circulating blood volume and 2) the «lipid» fraction, the total distribution space of which is unknown. We therefore preferred to express the absorbed activity at a certain time in terms of common units, *i.e.* in per cent of the dose per litre of blood. Furthermore, Grossman and Jordan (6) found no negative correlation between body weight and the blood level of radioactivity, and they came to the same conclusion.

It is evident that the radioactivity in the whole blood and in the precipitated part at a certain time after ingestion of labelled fat represents quantitatively a minimal part only of the absorbed radioactivity and qualitatively a fragment of a complicated process which includes at least absorption, deposition, destruction and deiodination of fat and, further, diffusion and excretion of liberated iodide. Blood radioactivity may give some clue to the absorptive function of the gut, but no quantitative data can be obtained from it alone. In an attempt to find a basis for the calculation of the total radioactivity absorbed it seemed feasible, therefore, to study more closely the behaviour of the total and precipitable blood radioactivity and the urinary excretion over a longer period. The results of these studies will be published in a subsequent paper.

SUMMARY

The absorption of I^{131} -labelled olive oil was studied in 6 patients with chronic pancreatic disease, in 3 patients with idiopathic steatorrhoea and in 27 patients who had had a previous attack of acute pancreatitis. Twentyone patients without gastrointestinal disturbances served as controls.

The radioactivity in whole blood and in the trichloracetic acid precipitate was measured 4, 5, and 6 hours after ingestion of a test meal containing between 20 and 50 μ C of labelled olive oil. The urinary excretion of radioactive iodine was measured during a 6-hour period.

The total blood radioactivity remained below the range of the distribution of the controls in the 3 patients with idiopathic steator-

rheoa and in 4 out of 6 subjects with chronic pancreatitis. The radioactivity in the precipitate from the control and pathological cases showed an appreciable overlapping. Those cases which had had an attack of acute pancreatitis gave values within the distribution range of the controls.

Potential errors affecting the method are discussed and the possibility of a quantitative calculation of the total amount of radioactivity absorbed is suggested.

The writers wish to express their gratitude to Miss M. Nummelin and Mrs. S. Jokinen for technical assistance.

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FROM THE MEDICAL DEPARTMENT, MARIA HOSPITAL AND THE FOURTH
DEPARTMENT OF MEDICINE, UNIVERSITY OF HELSINKI

STUDIES WITH I¹³¹-LABELLED FAT¹

II

SERIAL DETERMINATIONS OF BLOOD AND URINE RADIOACTIVITY
AFTER INGESTION OF I¹³¹-LABELLED OLIVE OIL

by

B.-A. LAMBERG, M. SIURALA and K. TURULA

(Received for publication August 4, 1959)

The use of I¹³¹-labelled fat for the detection of abnormalities of fat absorption was discussed in a previous paper (1). It was pointed out that the measurement of radioactivity in the blood at a certain time after a fat test meal may give some information on the intestinal absorption of fat, but that the values so obtained do not provide the necessary basis for definite conclusions regarding the amount of fat absorbed. It was considered possible, however, to carry out recovery calculations from the radioactivity in the whole blood, in the trichloracetic acid precipitable fraction of the blood and in the urine.

In order to establish a reasonable foundation for such calculations and the most suitable time after a test meal for their performance a study was made with serial determinations over a 24-hour experimental period of the radioactivity in blood and urine in persons without evident gastrointestinal disturbances.

MATERIAL AND METHODS

Tests were performed on 21 patients hospitalized for various diseases without any signs of malabsorptive disorders.

¹ This study was aided by a grant from the Sigrid Jusélius Foundation.

I^{131} -labelled olive oil was used. The methods used have been described in a previous paper (1).

Blood samples were drawn 2, 4, 6, 8, 10 and 24 hours after the test meal. The radioactivity in the whole blood and in the trichloroacetic acid (TCA) precipitable fraction of whole blood was measured. Urine was collected during corresponding periods. The radioactivity in the stools of 5 patients was measured.

RESULTS

The results of the individual radioactivity determinations in whole blood and in the TCA precipitable fraction of whole blood are presented in Fig. 1 and 2. The mean curves of the blood, precipitate and urine values are plotted on a semilogarithmic scale in Fig. 3. The blood values are expressed in per cent of the dose per litre, the urine values in per cent of the dose per hour and as the cumulative excretion in per cent of the dose.

The radioactivity in the blood behaved fairly uniformly in all the cases. After a rather steep rise in the curve, a peak value was usually reached 4 hours after the test meal. Between 4 and 6 hours there was a fairly steep drop in the curves, after which there was a change and the decline assumed a diminished rate. In principle, the same pattern was seen when the precipitate radioactivity and the excretion rate were plotted in the same way (Fig. 3).

From Fig. 3, in which the values are plotted on a semilogarithmic scale, it appears that some kind of equilibrium between the different radioactivity containing compartments was reached about 6 hours after the test meal. The half-time of the blood curves, whole blood and precipitate, and the excretion rate were estimated graphically to be 9.6, 9.0 and 7.6 hours respectively during the period 6 to 24 hour as a whole. Between 6 and 10 hours there was a decline in the blood curves and the corresponding half-time values during this period were 6.0, 5.0 and 7.6 hours, respectively.

DISCUSSION

To calculate the absorbed amount of fat, i.e. to make recovery calculations, it was necessary to develop a suitable working model. The reasoning was as follows:

From the gut the radioactive fat enters the *plasma lipid compartment*.

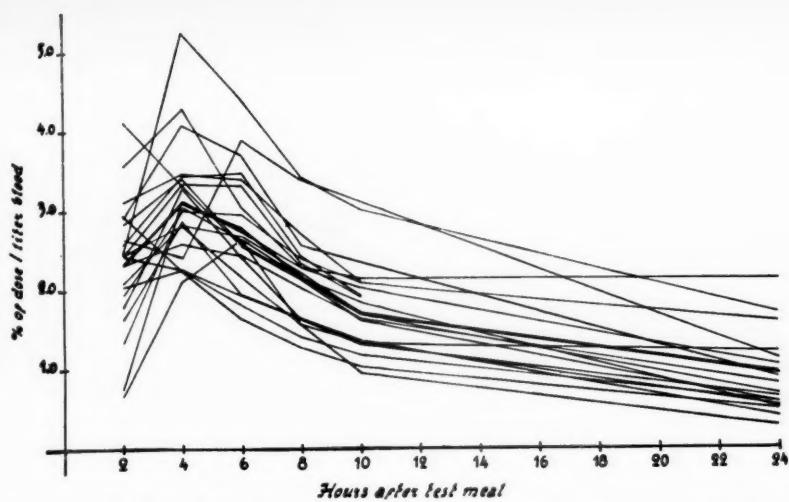


Fig. 1. — Radioactivity in whole blood after ingestion of I^{131} -labelled olive oil.

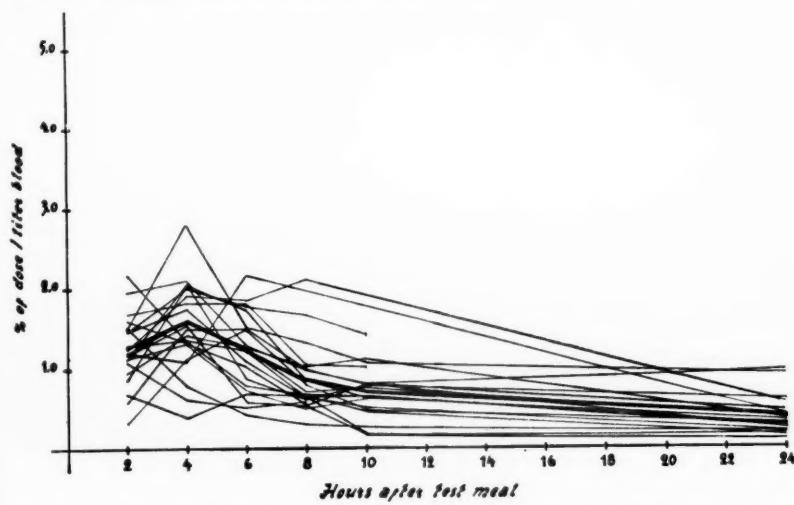


Fig. 2. — Radioactivity in the TCA precipitable fraction of whole blood after ingestion of I^{131} -labelled olive oil.

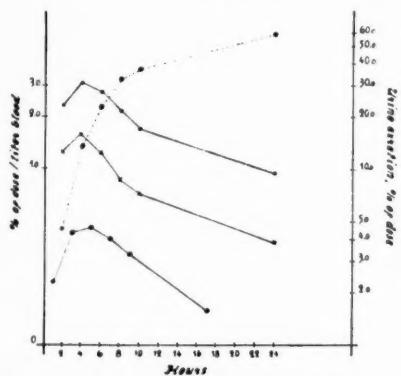


Fig. 3. — The mean curves of the radioactivity in whole blood (●—●, per cent per litre), the TCA precipitable fraction (x—x, per cent per litre), the cumulative urinary excretion (o---o, per cent of dose) and the urinary excretion rate (o—o, per cent of dose per hour) after ingestion of I^{131} -labelled olive oil.

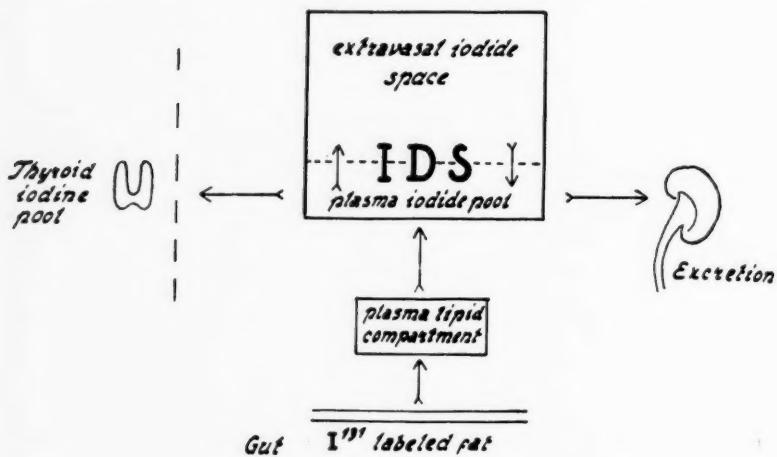


Fig. 4. — A simplified model of the distribution of radioactivity after ingestion of I¹³¹-labelled fat. IDS = iodide diffusion space.

ment. Throughout this process iodine is constantly liberated from the lipid bonds and it enters the *plasma iodide pool* as iodide. Provided the thyroidal uptake of iodide is blocked the liberated radioactive iodine in the plasma is equilibrated with the *extra-vascular iodide space*, both together forming the *iodide diffusion space* (IDS). From the plasma iodide is constantly excreted in the urine. This model, presented in fig. 4, most probably represents an oversimplification of the rather complex events, which include absorption, distribution and deiodination of fat and distribution and excretion of liberated iodide. As a working hypothesis, however, it may serve its purpose. Provided the calculations are performed at a time when the absorption from the gut has ended, the formula for recovered radioactivity would be:

$$(L \times Bv) + \left(\frac{(T-L)}{1-Hct} \times \text{IDS} \right) + E = \text{absorbed fat in per cent of the dose when}$$

L = lipid radioactivity in per cent of the dose per litre blood,
 Bv = blood volume, litres

$L \times Bv$ = circulating lipid radioactivity

T = total whole blood radioactivity in per cent of the dose/litre
 IDS = iodide diffusion space, litres

$\frac{(T-L)}{1-Hct} \times IDS$ = radioactivity in *IDS*, per cent of dose, in which
 $\frac{(T-L)}{1-Hct}$ represents the radioactivity concentration in the plasma
 supernatant and *Hct* hematocrit expressed as a fraction,
E = urinary excretion, per cent of the dose

Provided the calculations are made at a time when absorption has ended the fraction of the dose absorbed = 1-fecal fat not absorbed. When comparing two groups it does not, of course, matter whether the absorption has ended or not provided a state of equilibrium has been attained and possibly deposited radioactivity is reflected in some plasma fraction. A suitable time for the calculations seemed to be the 6-hour point. At this time, according to Fig. 3, some kind of equilibrium seems to have been established. According to the data presented by Borgström (2) and by Lundh (3), the absorption in normal persons is probably nearly 100 per cent 6 hours after the test meal. This statement is at least partly corroborated by the present data as indicated by the form of the curves in Fig. 1-3.

In addition to measurement of the radioactivity, the other factors needed can either be measured directly or calculated from the body weight. The blood volume can be calculated from the body weight or measured directly in several ways. The same applies to the iodide diffusion space which is some 25-35 per cent of the body weight and can be measured with a tracer dose of radioactive iodide in a separate experiment.

The practical use of this model rests, however, on certain assumptions. The two most important are 1) that the difference between whole blood radioactivity and the precipitate radioactivity represents only inorganic iodide and 2) that the circulating precipitate radioactivity represents at least the major part of the total lipid pool. The precipitation of whole blood with TCA, as used by Beres and coll. (4), may include, however, at least two sources of error: a) the coprecipitation of inorganic I^{131} and b) the presence of radioactivity bound to the erythrocytes. Before employing the model described above it was considered important to investigate these objections. The results will be presented in a subsequent paper (5). The most logical approach would be, of course, to use

plasma radioactivity measurements, especially for the determination of the radioactivity within the iodide diffusion space.

SUMMARY

Serial determinations of the radioactivity in whole blood and in a trichloracetic acid precipitate of whole blood and of the urinary excretion of radioactivity during a 24 hours experimental period after a test meal containing I^{131} -labelled olive oil were performed on 21 patients without evidence of gastrointestinal disorders.

The individual blood curves showed a fairly consistent pattern: After an initial steep rise a peak value was attained approximately 4 hours after the test meal. Between 4 and 6 hours a fairly steep decrease was seen, after which there was a change in the slope and a retarded rate of decline. After 6 hours some kind of equilibrium between different compartments seemed to have been reached. The data seemed to corroborate the view that absorption ended at this time.

It was suggested that determination of the total amount of absorbed fat may be carried out 6 hours after the test meal. A simplified model of the events was presented and the possibilities of recovery calculations discussed.

Acknowledgements. — The writers wish to express their gratitude to Miss M. Nummelin and Mrs S. Jokinen for technical assistance.

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STUDIES WITH I¹³¹-LABELLED FAT¹

III

CALCULATION OF THE TOTAL AMOUNT OF RADIOACTIVITY ABSORBED

by

M. SIURALA, B.-A. LAMBERG, K. TURULA and A. HARJANNE

(Received for publication August 4, 1959)

In previous studies (3, 4, 6) it was suggested that the total amount of I¹³¹-labelled fat absorbed from the intestine may be calculated from blood and urinary data at a certain time after the ingestion of a test meal. A model was presented (4) in which the circulating lipid radioactivity, the radioactive iodide present in the iodide diffusion space and the radioactivity excreted through the kidneys were taken into account.

Six hours after the test meal was regarded as a suitable time to perform the calculations as the absorption has probably ended by then. Estimation of the obviously most important pool, the iodide diffusion space, rested on the assumption that the radioactivity not precipitable with trichloracetic acid did represent the concentration of radioactive iodide within this compartment. The precipitation, however, was done from whole blood, according to Beres *et al.* (1) which may include important sources of error. Before using the model presented this point had to be checked. The aim of the present study, accordingly, was: firstly, to find out the errors inherent in the precipitation method and, secondly, to test the model in a clinical material.

¹ This study was aided by a grant from the Sigrid Jusélius Foundation.

METHODS AND MATERIAL

1. *Studies of the Precipitation Method.* — Two samples of blood were drawn from 5 patients without gastrointestinal disorders 6 hours after ingestion of I^{131} -labelled olive oil.

In one sample the whole blood radioactivity was measured after which precipitation with trichloracetic acid (TCA) was carried out as previously described with carrier iodide added. The radioactivity in the precipitate was determined and the difference calculated.

After measurement of the whole blood radioactivity the other blood sample was centrifuged and the plasma and the erythrocytes were separated. The radioactivity in the plasma was measured, after which the lipid-bound radioactivity was precipitated with TCA and the radioactivity in the precipitate counted.

In 4 cases the radioactivity in the separated erythrocytes was measured before and after one and two washings with isotonic saline. After the second washing, the erythrocytes were suspended in isotonic saline, hemolyzed with saponine and a TCA precipitation was carried out. The radioactivity in the precipitate was counted.

For the calculations in these experiments an average hematocrite of 0.375 was used.

The data in Table 1 should be analyzed individually rather than in terms of average values. There were fairly large individual variations. Some correlations should be recognized:

1) Obviously the whole blood supernate activity should be = plasma supernate activity. Some variations are seen in Table 1, especially in case 1 where a rather large difference is observed and the concentration is significantly higher in the WB supernate.

2) The difference: whole blood activity minus plasma activity should be = that remaining in the erythrocytes. Calculated in this way, a fairly large amount of radioactivity remained in the erythrocytes the range

TABLE I
DISTRIBUTION OF RADIOACTIVITY IN WHOLE BLOOD, PLASMA AND TRICHLORACETIC ACID PRECIPITATES

Case No.	Whole Blood			Plasma				Whole Blood Minus Plasma (Corr.) Conc.	Whole Blood Minus Plasma plus Plasma TCA ppt. Conc.	
	Cone., %/Litre	TCA ppt., %/Litre Blood	Supernate, %/Litre Blood	Cone., %/Litre	Corr., %/Litre	TCA ppt., %/Litre	Corr., %/Litre Blood			
1	3.08	0.52	2.56	3.53	2.21	0.39	0.24	1.97	0.87	
2	3.36	1.56	1.80	4.50	2.81	1.36	0.85	1.96	0.55	
3	2.55	0.64	1.91	3.01	1.88	0.30	0.19	1.69	0.67	
4	2.68	1.13	1.55	3.21	2.00	0.59	0.37	1.63	0.68	
5	2.05	0.69	1.36	2.61	1.63	0.31	0.19	1.44	0.42	

varying from 0.42 to 0.87 per cent of the dose per litre of blood, *i.e.* some 20—30 per cent of the radioactivity in the blood in spite of two washings with TCA and the use of adequate amounts of carrier iodide.

The radioactivity in the erythrocytes evidently consists of a) lipid bound radioactivity, b) inorganic radioactive iodide in the intracellular iodide space which is equilibrated with the plasma iodide pool, and c) adhered plasma radioactivity. In the experiment with hemolyzed erythrocytes it was found that only 0.17 per cent of the dose per litre of blood on an average was present in the erythrocytes precipitate. This means that about 0.25 to 0.70 per cent of the dose per litre of blood should be distributed between compartments b) and c).

3) Whole blood precipitate activity should = plasma lipid activity + the radioactivity in the erythrocytes, *i.e.* equal to the sum of the plasma TCA precipitate activity and the whole blood activity minus the plasma activity (last column in Table 1), when whole blood minus plasma activity represents the erythrocyte activity. In this respect, too, there were discrepancies, especially in case 1. Such variations may be due to the variable release of iodine from the erythrocytes, variable adhesion of plasma during the precipitation procedure and variable co-precipitation of iodine. This is probably also the reason for the discrepancies mentioned in paragraph 1 between the whole blood and plasma supernates.

The following conclusions may thus be drawn regarding the precipitation of whole blood: The radioactivity present in the whole blood precipitate does not represent the lipid radioactivity only. It also includes a rather large and variable fraction of radioactivity present in the erythrocytes and maybe co-precipitated radioactivity. Hence, the whole blood supernate after TCA precipitation cannot either be regarded as representative of the iodide concentration in the iodide diffusion space.

These conclusions were also substantiated by the extremely variable recovery data obtained from calculations of values presented in previous papers. It was concluded that the most logical approach for an adequate solution would be to use plasma and plasma TCA precipitate to represent the blood lipid pool and the iodide diffusion space.

2. *The Patients.* — Ten patients without evidence of malabsorptive disorders and 3 patients with idiopathic steatorrhoea were studied. In accordance with previous considerations, blood samples were drawn 6 hours after the test meal (3, 4).

3. *The methods* were in the main the same as those previously described except for the use of plasma instead of whole blood.

The 6-hour iodid diffusion space was calculated in 8 cases from the body weight, assuming that it is an average of about 36 per cent of the body weight (5). In 5 cases it was measured in a separate experiment. The thyroid uptake of radioactive iodide was blocked in the same way as previously described. 6 hours after a tracer dose of radioactive iodide given orally, the plasma and urinary radioactivity were recorded. The per cent of the dose retained in the body was calculated and divided by the plasma concentration, which gave the iodide diffusion volume in litres.

TABLE 2
RADIOACTIVITY IN PLASMA, PLASMA TGA PRECIPITABLE FRACTION, URINE, IODIDE DIFFUSION SPACE AND THE TOTAL RECOVERY OF RADIOACTIVITY 6 HOURS AFTER THE INGESTION OF ^{131}I -LABELLED OLIVE OIL

	Diagnosis	Sex	Age	Weight	Volume in Liters	Content, % of Dose	LD.S. 6 hrs.		Plasma 6 hrs.		Urine Excretion 6 hrs., % of Dose	Recovery % 6 hrs., % of Dose	
							Conc., % of Dose/l.	Precipit., Conc. % of Dose/l.	Volume in Liters	Precipit., Content % of Dose			
1.	Myocardial infarction	♀	65	75.5	23.00*	2.22	51.00	3.40	0.48	1.63	2.70	17.60	70.20
	Congestive heart failure	♀	74	62.0	20.10*	2.49	50.10	2.80	0.39	1.09	2.88	10.40	61.60
2.	Congestive heart failure	♀	60	68.0	23.11*	2.49	57.54	3.06	0.86	2.65	3.35	13.23	73.40
3.	Pneumonia												
	Extrarenal azotemia												
4.	Gastric ulcer	♂	60	65.0	33.52*	2.01	67.38	3.30**	0.66	2.18	2.67	10.76	80.32
	Pulmonary emphysema												
5.	Nephrosclerosis	♂	36	87.4	27.85*	2.19	60.99	4.25**	0.41	1.74	2.60	12.53	75.26
	Cardiosclerosis												
6.	Hodgkin's disease	♂	44	67.6	24.34	2.62	63.77	3.04	0.59	1.80	3.21	7.61	73.18
	Pulmonary tumor	♂	65	55.6	20.02	2.71	54.25	2.50	0.30	0.75	3.01	13.56	68.56
7.													
8.	Myocardial infarction	♂	66	80.5	28.98	2.30	66.65	3.62	0.31	1.12	2.61	15.34	83.11
	Hypertension												
9.	Myocardial infarction	♂	53	70.0	25.20	3.14	79.13	3.15	1.36	4.28	4.50	20.01	103.42
	Pulmonary emphysema												
10.	Methyl alcohol poisoning	♂	38	66.7	24.01	3.14	75.39	3.00	0.39	1.17	3.53	16.15	92.71
11.	Steatorrhoea	♀	15	45.0	16.20	1.84	29.21	2.02	0.20	0.41	2.04	5.53	35.15
12.	Steatorrhoea	♂	53	50.0	18.00	0.98	17.68	2.73**	0.45	1.23	1.43	9.45	28.36
13.	Steatorrhoea	♂	29	56.3	20.27	0.84	17.00	2.53	0.08	0.21	0.92	8.13	25.34

The plasma was precipitated with TCA, but no significant amount of radioactivity was found in the precipitate.

The blood volume was calculated from the body weight (7.2 per cent) and the plasma volume according to the hematocrit (0.375). In 3 cases the blood and plasma volumes were measured with Evans blue.

4. *The calculation of the accountable radioactivity* was carried out in accordance with the model and formulae presented previously (3):

$(L \times Pv) + ((T-L) \times IDS) + E =$ accountable radioactivity in per cent of the dose and assumed to be = fat absorbed, when

L = plasma lipid radioactivity, in per cent of dose/litre

Pv = plasma volume, litres

T = plasma total radioactivity in per cent of the dose/litre

IDS = iodide diffusion space, litres

E = radioactivity excreted in the urine in per cent of the dose and

$(L \times Pv)$ = circulating lipid-bound radioactivity in per cent of the dose,

$(T-L)$ = plasma iodide in per cent of the dose/litre

$(T-L) \times IDS$ = radioactive iodide in the IDS in per cent of the dose.

RESULTS

All the data are presented in Tables 2 and 3. It is seen that the radioactivity present in the IDS does indeed represent a fairly

TABLE 3

RADIOACTIVITY IN WHOLE BLOOD 4, 5 AND 6 HOURS AFTER THE INGESTION OF I^{131} -LABELLED OLIVE OIL

	Whole Blood, %/Litres			Whole Blood Precipitate, %/Litres		
	4 hrs.	5 hrs.	6 hrs.	4 hrs.	5 hrs.	6 hrs.
1.	2.69	2.51	2.39	0.86	1.25	1.45
2.	2.47	2.45	2.44	1.71	1.18	1.46
3.	2.59	2.41	2.25	1.36	1.04	1.19
4.	2.78	—	2.16	1.36	—	0.96
5.	2.22	—	2.18	1.08	—	0.97
6.	1.41	—	2.74	0.73	—	1.10
7.	2.07	—	2.55	0.63	—	0.64
8.	2.14	—	2.05	0.76	—	0.69
9.	4.19	—	3.36	1.98	—	1.56
10.	3.90	—	3.08	0.93	—	0.52
11.	1.91	—	1.91	0.92	—	0.80
12.	1.42	1.21	1.25	0.24	0.62	0.45
13.	0.86	—	0.77	0.11	—	0.15

large fraction of the dose given, varying in the control cases from 50.1 to 79.13 per cent. One of the steatorrhoea patients had plasma precipitable activity values within the control range. The total plasma activity, however, was below the lowest in the control group in all the steatorrhoea cases.

The most striking difference was seen in the values of total accountable radioactivity. The recovery varied in the control cases from 61.6 to 103.42 per cent of the dose, whereas the values in the patients with steatorrhoea were 25.3, 28.4 and 35.2 per cent, respectively. A certain correlation seemed to exist between the accountable radioactivity and the total plasma activity obviously because the latter is the major determinant of the radioactivity in the IDS, the lipid radioactivity being fairly low.

DISCUSSION

From the present studies on the lipid precipitation from whole blood and from plasma it appeared that the former method may involve rather important sources of error, as has been pointed out by Turner (8). The supernate after TCA precipitation of whole blood does not represent that part of the inorganic iodide diffusion space which the plasma supernate should do. The principal sources of error in whole blood precipitation are probably 1) variable release of intracellular iodide during the precipitation procedure, 2) adhesion of radioactive plasma to the erythrocyte sediment, and 3) the co-precipitation of radioactivity.

Calculation of accountable radioactivity 6 hours after a test meal containing radioactive fat was based on the assumption that the plasma supernate represents part of the IDS. The accountable radioactivity as representing the fat absorbed was calculated according to a model previously described in which 1) the radioactivity excreted through the kidneys, 2) the radioactivity in the circulating plasma lipid pool and 3) the radioactivity in the IDS were added. The calculations were made from values obtained 6 hours after the test meal. The choice of this time and the assumptions inherent in the calculation method have been discussed previously (3, 4). In these calculations, the concentration of radioactivity in the supernate after TCA precipitation of plasma was regarded as representing the iodide equilibrated within the whole IDS.

When analyzing the data obtained from the present material it was observed that the circulating plasma lipid pool, 6 hours after the test meal, contained only 0.5 to 1.7 per cent of the dose administered. The plasma lipid concentrations were accordingly extremely low. Thus it was not very surprising to find that one of the steatorrhoea patients had a plasma lipid value within the control range. It may be concluded from this that measurement of the plasma lipid concentration or of the circulating lipid alone cannot be regarded as very informative.

Further substantiation for this view is the fact that from 50 to 79 per cent of the radioactivity administered was located in the IDS. This value was very high and corroborated the authors initial idea that this compartment represents a fairly important site of location of the absorbed radioactivity. Even slight variations in such a large pool may be of great importance for the blood and plasma concentration and for the accountable radioactivity.

Although the material is small, there was a fairly striking difference between the steatorrhoea patients and the control group as regards recovered radioactivity. A rough correlation of sorts seemed to exist between the total plasma concentration and the recovered radioactivity. The reason for this is that the iodide in the IDS is a determinant of the total plasma concentration.

From the recovery values it is apparent that a significant amount of radioactivity cannot be accounted for, not even if the fecal excretion is taken into account. Assuming that this is normally 0—4 per cent of the dose there is still some 20—30 per cent not accountable for in many cases. There are several explanations. For instance, some radioactivity may still remain in the intestinal lumen at this time, in the intestinal mucosa or in the lymph, or the unaccountable part may have been deposited. The last possibility cannot be disregarded. Deposition could take place in the metabolic lipid pool of the liver in which the formation and deiodination of lipoproteins presumably does take place. In addition to this physiological deposition, also pathological deposition must be taken into account (2).

Thus it seemed to be of interest to continue these investigations with special reference to the deposition of radioactivity. These studies, with I¹³¹-labelled triolein over an extended experimental period, will be published in a subsequent paper (7).

SUMMARY

The validity of the trichloracetic acid precipitation of whole blood for the measurement of blood lipid concentration was studied in 5 patients after ingestion of I^{131} -labelled olive oil. It was found that the method involves several potential sources of error and that the radioactivity of the precipitate cannot be regarded as representative of the blood lipid radioactivity. It followed also that the radioactivity in the TCA supernate does not either represent the concentration in the iodide diffusion space. It was suggested that the concentration of lipid and iodide radioactivity be determined from plasma.

Using this precipitation method, an attempt was made to calculate the accountable radioactivity (except feces) in the body in 10 control cases and 3 patients with idiopathic steatorrhoea 6 hours after a test meal containing I^{131} -labelled olive oil. The plasma lipid radioactivity was found to represent a fairly small and variable pool, whereas the radioactivity in the iodide diffusion space represented the greatest pool in which radioactivity was located. The accountable radioactivity showed fairly good separation between the controls and the pathological cases and there seemed to be some correlation between the total plasma radioactivity and the recovered radioactivity. The importance of the iodide diffusion space as a site of concealment of the major part of the absorbed radioactivity is stressed.

6 hours after the test meal there was still a significant amount of radioactivity unaccountable. The possibilities of this fraction being deposited somewhere are discussed.

The writers wish to express their gratitude to Miss M. Nummelin and Mrs S. Jokinen for technical assistance.

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EFFECT OF URAEMIC HUMAN BLOOD ON MICE

by

EEVA-LIISA KOSKELI

(Received for publication September 1, 1959)

There have been attempts to remove the toxic substances which collect in blood when the function of the kidneys fails by means of different kinds of artificial kidneys. For this purpose several researchers have been experimenting with ionexchange resins. Szold and Vanja (1) have shown in their rather detailed paper that in their method no other essential change happens in the composition of blood but the reduction of the NPN-substances by about 50 per cent.

Some Hungarian research workers (2) decided to experiment with mice as laboratory animals if the toxicity of uraemic blood was really reduced if treated with resins. Blood was taken from dogs which had been subjected to bilateral nephrectomy 48 hours earlier. They noticed that, on the contrary, blood treated with the resins Dovex-50, Dovex-2 and IR 4B became very toxic to mice. They injected 1 ml of uraemic blood which had streamed through the resin into mice intraperitoneally every day during five days. In the coarse of this observation 86 of the 100 animals used died. On the other hand, only 22 mice died of the 84 which got untreated uraemic blood. The resins seemed to have no essential difference.

The same method was used in this work to find out if uraemic human blood gives similar results. Heparinised plasma of twelve uraemic patients was collected. Anamnestically and clinically unmistakable kidney disease and NPN-rate over 50 mg per cent were hold as criterions. The plasmas were allowed to stream very slowly through resin columns of 15 cm length and 1 cm diameter.

The resins had first been treated with heparinised Ringer solution to bring about the normal ion environment. Dovex-50 cation-exchange and Dovex-2 anion-exchange resins were used.

After this 0.5—1 ml of both treated and untreated uraemic plasma were injected intraperitoneally into mice of 20—30 gr weight every day during five days.

TABLE 1

	Uraemic Untreated Plasma	Uraemic Plasma Treated with Resin
Number of animals	12	15
Number of animals that died ..	0	5

All these five patients whose blood gave fatal results had very serious kidney failure. NPN-rates were 80, 99, 108, 110 and 294 mg per cent. All of them had low alkali reserve, anaemia, hypertension and proteinuria. The most uraemic of these bloods was injected in four mice in all; however, only one of these died.

As a control a corresponding series was made with the blood of quite healthy young persons.

TABLE 2

	Healthy Untreated Plasma	Healthy Plasma Treated with Resin
Number of animals	5	4
Number of animals that died ..	0	0

A series was also made with the blood of non-uraemic weak patients chosen at random.

TABLE 3

	Pathological but Nonuraemic Untreated Plasma	Pathological but Nonuraemic Plasma Treated with Resin
Number of animals	7	8
Number of animals that died ..	0	0

Consequently, this small material seems to support the results of the experiments of the Hungarian researchers. In the blood both of a uraemic dog and of a uraemic man there is some substance toxic at least to mice which is liberated in active form when treated with resins and which for the present is little known (3).

SUMMARY

Uraemic human blood streaming through Dovex-50 and Dovex-2 resin columns was noticed to become somewhat toxic to mice when injected intraperitoneally into them. Five out of 15 animals died. All 36 control animals survived.

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WOUND HEALING IN DENERVATED SKIN OF RATS

by

GUSTAF ELFVING

(Received for publication September 4, 1959)

The hypothesis that the nervous system plays a role in wound healing is of long standing. It is common knowledge that wound repair and recuperation in general are impaired, even hindered in the paralysed part of the body. It is difficult, however, in some respects impossible even, to make a more thorough experimental study of the problem in the absence of suitable and reliable methods. Severing or resection of the nerve responsible for the innervation of a certain area is not a sufficient measure. Ganglionectomy is a more radical procedure leading to more certain denervation.

There are several reported investigations which prove the significance of nerves in wound healing and regeneration especially in lower animals. Singer (7), for instance, found that it is the number rather than the origin of nerve fibrils that determines whether regeneration will or will not occur in adult *Triturus*. Thornton (9, 10, 11) showed in *Amblystoma* larvae that neural invasion is of decisive importance in the wound healing phase of regeneration and in regeneration in general. Ruben and Frothingham (6) demonstrated in larval *Amblystoma Opacum* and adult *Triturus Viridescens* that serial resections of the brachial plexi will render the host limbs incapable of responding to a traumatising implant of *Rana Pipiens* kidney by producing an accessory growth.

Denervation has been used in higher animals especially in carcinogenesis studies. The results have been contradictory in part. This is probably due to the denervation technique employed, its

possible inadequacy and, in addition, to other dysfunctions caused by the procedure such as changes in blood supply and mechanical irritation in the denervated areas.

By totally excising a segment of skin from the animal's back and then grafting it back into place as an autoplast Cramer (1) produced denervated skin in mice. On painting the autoplast and the control skin with coal tar he found the tumour to form more rapidly at the control site. Cramer (1) drew the conclusion that the presence of a peripheral nerve is indispensable for the production of the chronic irritation which leads to the development of cancer. Julius (4, 5) arrived at the contrary result when, using the same method, he found a couple of weeks after the operation that hyperneuria instead of denervation had developed in the autoplast. Itchikawa and Ookubo (3) altered the innervation of the rabbit's ear by cervical neurectomy or cervical sympathetic ganglionectomy or a combination of both procedures. On irritating the rabbit's ear subsequently they found a certain acceleration in tumour production. Tavares and Morais (8) obtained a contrasting result with the same technique.

By the unilateral resection of the lower six thoracic dorsal root ganglia in continuity with dorsal and ventral nerve roots and peripheral nerve trunks Hasson (2) produced in mice denervation in a band of skin. He found that methylcholantrene caused a more rapid appearance of tumours in denervated skin. One of the advantages of this method is that it precludes the nerve fibre regeneration, denervation is radical and the result can be regarded as reliable. The method obviously does not involve any alterations in the blood supply of the denervated area nor, apparently, in any other function, not least because the site of operation is far from the maximum of the denervated point.

The purpose of this study was to study wound healing in denervated skin of rats by Hasson's (2) denervation technique and punching wounds at the denervated site.

MATERIAL AND METHOD

General. -- White rats of Dervley-Sprague strain weighing 200—250 g were used in the tests. All the animals were kept on standard laboratory diet and in identical conditions. All the opera-

tions were performed at the same time of the day. The animals were divided into three groups of which the first and the second served as controls and the third as the actual test group.

Anesthesia. — Prolonged deep anesthesia was necessary because the surgical procedure required from 1½ to 2 hours. Abbot's Nembutal was used as the narcotic in a dilution of 6 mg per cc. 4—5 mg/100 g of body weight was injected introperitoneally. Deep anesthesia was generally produced in 15 minutes and its duration ranged from 45 minutes to nearly 1½ hours. If necessary a fourth of the above dosage was injected as a supplementary dose. Not a single animal was lost in narcosis.

Surgical Technique. — (a) *1st control group* (pseudo-operation): The hair was carefully removed from the back and part of the flanks of the test animal with small scissors. The animal was placed on its stomach, the paws spreadeagled. An incision of about 6 cm long was made in the skin along the midline of the back over the thoracic curvature of the vertebral column. The deep fascia including the interscapular fat was divided longitudinally without injuring the blood vessels. The divided fascia was freed laterally and the exposed outer muscular coat was split with small scissors through the tendinous portions along the lateral margins of the vertebral arches. This split muscle was also drawn to the side, together with the fascia and the skin. The deeper muscular coat thus exposed, which covers the ribs and the intercostal spaces, was split in the direction of and between the ribs with scissors after the animal had been turned into its left flank. To protect the intercostal artery these incisions were made close to the next rib caudally. This was done between the six most caudally situated ribs after counting from the lowest, the 12th.

With the intercostal nerves thus visible the surgical procedure in the first control group came to an end. The innermost muscles were replaced. The outer muscular coat was sutured with gut, likewise the deep fascia. The skin was closed with silk.

(b) *2nd control group* (cutting the intercostal nerves): the surgical procedure was the same in this group except that the exposed intercostal nerves were cut. The wounds were then closed as in control group 1.

(c) *the actual gangliectomy group:* gangliectomy was performed according to Hasson's (2) method (Fig. 1). In this group the surgical

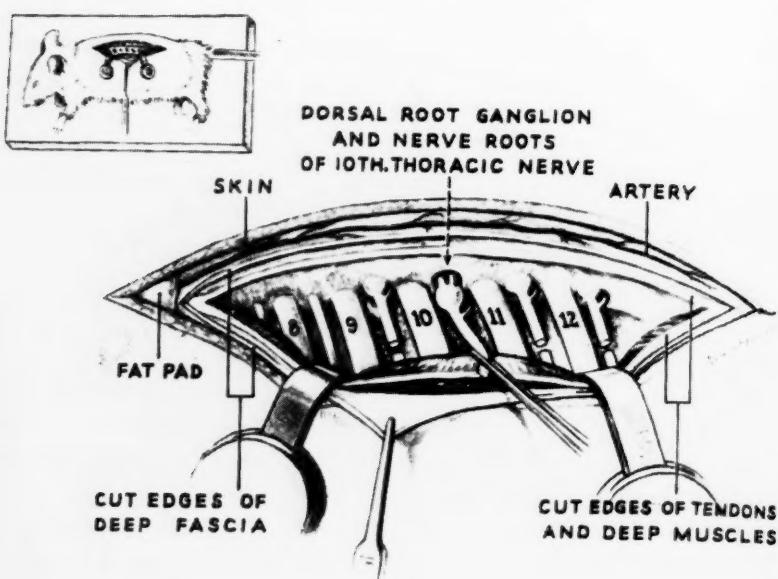


Fig. 1.—Surgical Technic, composite drawing. Thoracic nerves 7 and 8 illustrate the appearance of the exposed and mobilized peripheral nerve trunks. The 9th, 11th, and 12th thoracic nerves demonstrate the aspect of the exposed and mobilized dorsal root ganglia. The 10th thoracic nerve illustrates the appearance of the peripheral nerve trunk in continuity with the dorsal root ganglion and dorsal and ventral nerve roots just prior to section of the nerve roots and excision of these neural elements en bloc. — (From the paper by Hasson in Cancer Research 1958;3:267. — My thanks are due to H.P. Rusch, Editor-in-Chief, and to J. Hasson for permission to use this illustration.)

procedure after the intercostal nerves were exposed was performed under a double magnifying glass. Hasson (2) had to use a greater magnification when operating on mice. Apart from this and the selection of instruments used, the surgical procedure followed adhered closely to his method.

The intercostal nerves were freed over a distance of c. 1—1½ cm as close as possible to the intervertebral foramen. The parietal pleura was exposed and easily punctured in mobilising the nerves, with resultant fatal pneumothorax. In one case the pneumothorax was treated successfully with a muscle lambo and subsequent aspiration. If the nerve was exposed too near the intervertebral foramen, massive bleeding always ensued. Haemorrhage of this type, however, could usually be checked by compressing the site for some minutes with a minimal gauze pad dipped in saline.

The nerves were severed c. 1 cm from the intervertebral foramen. The peripheral part of the nerve was left intact, the central portion was followed up to the intervertebral foramen as far as a slight but distinct swelling. The swelling was a ganglion surrounded by loose connective tissue. By pulling carefully from the distal end of the central part of the severed intercostal nerve it was possible to free the ganglion and bring it into view, thus revealing also the dorsal branch once the ganglion emerged from its foramen. This was the most difficult and most time-consuming phase of the operation. Watchmaker's pincers ground down to a thickness of



Fig. 2. — The bulge of the abdomen after denervation on the right side. Note the irregularity of the 4 days old wounds.

0.5 mm proved of great assistance, likewise similarly treated scissors with which the ganglion was cut free sharply from the more central parts. The ganglion, the attached roots and the peripheral nerve trunk were excised en bloc. The same procedure was carried out on all the six lowest thoracic nerves. Haemorrhages were controlled as described previously.

The wound was closed in three layers in the same way as in the control groups.

The method caused unilateral denervation of a band of skin to the right. The animals displayed paralysis and flaccidity of the abdominal muscles, causing a characteristic bulging of the right side of the abdomen (Fig. 2).

Applications of Wounds: skin was removed under ether from all the test animals 2 and 4 weeks postoperatively on both sides of the abdomen with a round skin punch 8 mm in diameter. In the actual denervation group the wounds were punched to the right, i.e. on the denervated side where the abdominal bulge due to denervation was most pronounced. On the contralateral side the punching was done at the corresponding site located as accurately as possible with a pair of dividers. The control groups also were punched at the corresponding points.

After punching, the isolated skin flap was removed with anatomical scissors and blunt scissors without damaging the subcutaneous tissue. If the first wound failed to heal in two weeks a new wound was made in healthy tissue 4 weeks after the original operation. The site of the new wound was in the bulging part of the abdomen of the denervated animals.

Criteria of Wound Healing. — The following criteria were observed in wound healing: (1) the size of the wounds. The wounds were traced on fixed days under ether through thin tissue paper. The traces were planimetered in tenfold projection with a projector. (2) Attention was paid to the number of wounds which varied in the denervated group. (3) The shape of the wounds was observed direct from the traces. (4) The wound healing time was recorded.

RESULTS

General. — Before the tests proper were started some ten animals were used experimentally to find suitable instruments and to master

the operating technique. Five rats were operated on in each of the three test animal groups. In the first control group one rat died for unknown reasons two weeks postoperatively. One animal in the second control group suffered the same fate.

In the actual denervation group all the animals survived for 6 months when they were sacrificed for other examinations.

Abdominal Bulging. — Pronounced abdominal bulging was observed in the abdomen of all of the denervated animals on the operated side immediately after the operation. No corresponding alteration was established in a single animal of the control group. This bulge was seen best when the test animal was suspended by its tail. The explanation of this phenomenon, mentioned by Hasson, is not known. Hasson (2), however, regarded it as a criterion of successful denervation surgery. The bulge may be caused by paralysis of the skin muscles.

As late as six months after the operation the abdominal bulging in question was still as clearly visible in all the animals as immediately postoperatively.

Healing of Operation Wounds. — Except for one animal of the denervation group, all the operation wounds healed by first intention. In the one exception a high card-like induration, $\frac{1}{2} \times \frac{1}{2}$ cm, developed approximately in the middle of the incision. It healed spontaneously in three weeks.

Healing of Applied Wounds. — Apart from the healing of the punched wounds of denervated rats, seen in Fig. 3, the wound healing in the control groups is illustrated in Figs. 4 and 5. The traces are of the wounds applied two weeks after the actual operation. The figures also show the variation in wound size and healing time, and possible additional wounds and lesions. Table 1 gives the planimetry results of the punched wounds of all the test animal groups.

Size of the Wounds: A fairly evenly diminishing size was seen in both the control groups and on the control side of the denervation group. On the other hand, the size of the wounds varied considerably on the denervated side. The planimetry table reveals clearly how the size of the wound area showed differences in reduction and enlargement before the final healing. All the wounds healed finally.

Number of Wounds: Although only one round wound was applied on either side it was found that »division» of the wound occurred

Days after appli- cation of wounds	Rat 1		Rat 2		Rat 3		Rat 4	
	Oper- ated side	Normal side	Oper- ated side	Normal side	Oper- ated side	Normal side	Oper- ated side	Normal side
3	o	o	l	o	o	o		
5	o	o	l	o	l	-	o	o
7	o	o	l	o	l			
10	o o	o	l	o	l			
12	o o	o	l		l		o	o
14	o o		l		l			
16	o		l					
19			l		l			
28					l		o	o
40					l			

Fig. 3. — Wound healing in gangliectomized rats.

Days after appli- cation of wounds	Rat 10		Rat 11		Rat 12		Rat 13		Rat 14	
	Oper- ated side	Normal side								
3	○	○	○	○	○	○	○	○	○	○
5	○	○	○	○	○	○	○	○	○	○
7	○	○	○	○	○	○	○	○	○	○
10	○	○			○	○	○	○	○	○
12	○				○					
14	○									

Fig. 4. — Wound healing in pseudo-operated rats. (Control group I).

Days after appli- cation of wounds	Rat 20		Rat 22		Rat 23	
	Oper- ated side	Normal side	Oper- ated side	Normal side	Oper- ated side	Normal side
3	○	○	○	○	○	○
5	○	○	○	○	○	○
7	○	○	○	○	○	○
10	○	○	○	○		
14	○		○			
16			○			

Fig. 5. — Wound healing in rats after cutting the intercostal nerves. (Control group II).

TABLE I
THE AREAS (SQ.MM.) OF THE WOUNDS OF ALL ANIMAL GROUPS
Gangliectomized rats

Days after Application of Wounds	Rat 1		Rat 2		Rat 3		Rat 4	
	Oper- ated Side	Normal Side	Oper- ated Side	Normal Side	Oper- ated Side	Normal Side	Oper- ated Side	Normal Side
							133	
							103	
3	17	15	10	30	27	27	130	12
5	13	9	18	13	89	5	115	11
7	10	7	21	3	55		114	
10	9	3	67	3	60		86	
12	30	3	61		102		135	
14	11		60		65			
16	5		34					
19			25		100			
28					49		117	
40					135			

Pseudo-operated rats. (Control Group. 1)

	Rat 10		Rat 11		Rat 12		Rat 13		Rat 14	
3	31	18	34	16	15	8	34	9	26	35
5	29	9	26	5	5	25	19	17	11	20
7	19	5	10	8	4	26	18	5	10	12
10	11	1			2	5	4	3	5	4
14	6					1				
16	1									

Rats with cutted intercostal nerves (Control Group. 2)

	Rat 20		Rat 22		Rat 23	
3	44	28	55	42	43	49
5	14	13	49	11	17	16
7	15	4	33	6	7	7
10	7	3	17	3		
14	3		9			
16			4			

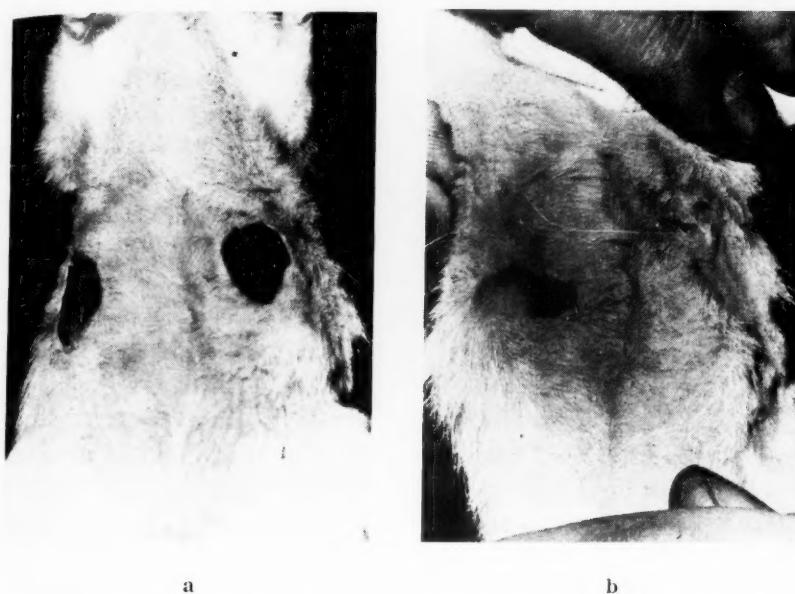


Fig. 6. — The punched wounds of one gangliectomized rat immediately after application (a), and 5 days later (b), when the control side had almost closed and the wound of the denervated side was still completely open.

sooner or later in denervated rats, on the denervated side only. However, neither the division of the wound nor the increase in the wound area affected the final healing.

Shape of Wounds: Initially round, the wounds later changed shape considerably in all the groups. The cutaneous muscles obviously play role in this process. For instance, in rat No. 3 of the denervation group the wound on the control side was almost completely closed 5 days after the punching while the contralateral side was still completely open (see Fig. 6).

Wound Healing Time. — The attention was attracted above all by the formation on the denervated side of an extensive lesion at the bulging point in rat No. 4 prior to punching. This wound connected with the punched wound ten days later to split up again into several wounds 24 days later.

The wounds of all the rats healed eventually. The last to heal were the wounds on the denervated side of the denervated rats in cases 3 and 4, 53 and 61 days respectively after the punching.

During the six months for which the rats were observed none showed later spontaneous wounds.

Mathematical treatment of the results of the basis of the planimetry table indicated that wound healing on the denervated side was highly significantly delayed ($P \leq 0.001$) from all the other wound healing processes. Another significant ($P \leq 0.01$) finding was that in the denervated group the wounds on the control side healed more rapidly than corresponding wounds in the other groups. However, because of the smallness of the groups it is probably not possible to draw more far-reaching conclusions from this significance.

DISCUSSION

Although there appears to be no literature on wound healing in denervated skin of mammalia and comparison is therefore impossible, retarded wound healing may in certain respects be compared with retarded carcinogenesis in similar circumstances (8). The results of such comparison, however, have been contradictory as regards carcinogenesis (4, 5). The divergency probably arises essentially from the nature of the denervation and is indicative of the difficulty of producing denervation.

The results of the present investigation substantiate clearly the view that wound healing is retarded in denervated skin of rats. The variable size and shape of the wounds on the denervated side and the fact that one animal developed a wound prior to actual punching are probably connected with the abdominal bulging caused by the operation. The bulging may cause the abdomen of the denervated side to touch the floor of the cage when the animal moves. But this does not eliminate the clear-cut results obtained. It must be remembered that all the wounds healed eventually and that none of the animals developed a subsequent wound during six months though the abdominal bulge remained unchanged.

The retardation in wound healing must surely be the work of several factors in addition to deficient or non-existent innervation. It seems probable that a considerable role is played by the changing metabolic conditions of the area in question and a general decrease in the ability to react.

It may be mentioned by way of conclusion that wound healing

in denervated rat skin is clearly slower than in a healthy animal. The innervation of skin is of great importance in wound healing. It is to be assumed that this finding will apply also to man although there are structural differences between human skin and rat skin that must be kept in mind.

SUMMARY

The healing of a wound caused by punching was examined in denervated rat skin. Denervation was produced according to Hasson's method by resecting the lower six thoracic nerve root ganglia in continuity with dorsal and ventral nerve roots and peripheral nerve trunks. Compared with different control groups, wound healing was found to be distinctly slower in denervated skin.

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FROM THE DEPARTMENT OF ANATOMY, THE DEPARTMENT OF PHARMACOLOGY
AND THE DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY, UNIVERSITY OF
TURKU, FINLAND

EFFECT OF AUDIOGENIC-VISUAL STIMULI ON PREGNANT RATS

by

K. SOIVA, M. GRÖNROOS and A. J. AHO

(Received for publication September 16, 1959)

Certain recent observations have led to studies concerning stress which are particularly interesting. According to Selye (10, 11, 12, 13, 14) pregnancy is capable of protecting the animal from some noxious effects of the treatment under certain conditions and also makes the animal more liable to these effects under some other conditions. It has also been found that psychic stress affecting the vegetative nervous system produces a reaction which differs from that caused by physical exercise (3). Psychic strain, *e.g.* an examination, can increase the secretion of adrenalin many times over normal in man, but the secretion of noradrenalin remains about normal; the adrenocortical activity is seldom increased (5).

Previous studies have shown that audiogenic stimuli produce changes in the weights of the endocrine glands (9) and audiogenic-visual stimuli organic and functional changes in the genitals of non-pregnant rats (15). The purpose of the work reported in the present paper was to find out what effects audiogenic-visual stimuli, which produce in non-pregnant rats an increase in the weight of the adrenals and a decrease in the weight of the pituitary glands and ovaries, and a reduction in gonadal function (determined by vaginal smear samples, histological picture in the pituitary glands and ovaries, and gonadotrophin and oestrogen excretion (15), produce on the weight of pregnant rats and on that of their adre-

nals, pituitary gland, ovaries, heart and kidneys, and on the development of the pregnancy, the weight of the litter and the blood pressure.

MATERIAL AND METHODS

The series consisted of female albino rats weighing 189—274 g, which were divided into two groups: a test and a control group. Ten animals made up the control group; five of them were at the age of 5 months and the others at the age of 8 months. The test series consisted of 15 animals, about a half of which had littered previously and were at the age of 8 months, the other half being at the age of 5 months and nulliparae.

At the beginning of the experiment the animals were weighed and placed into cages with a male in groups of 5 females. The onset of pregnancy was ascertained, the animal was weighed and transferred immediately into a single cage. The treatment was begun at the same time. It was largely similar to that described by Soiva *et al.* (15); yet no pain stimuli were made use of at all. The animals underwent an audiogenic-visual treatment twice a day, after which they were allowed to live in single cages under normal laboratory conditions. The bottoms of the cages were of a large-meshed wire netting which enabled the observation of any possible abortions. The controls lived under similar conditions although they were subjected to no audiogenic-visual stimuli. The blood pressure was taken every second day $\frac{3}{4}$ — $1\frac{1}{2}$ hours after the treatment given in the afternoon. The blood pressures of the controls were measured correspondingly. The measurement was done photoelectrically (using The Photoelectric Tensometer, Metro Industries, New York) on the right hind leg without narcosis as the animal was lying comfortably in a leather case. Three successive measurements were made every time (the difference between the various readings was 5—10 mm Hg) and the calculated mean of the readings was recorded.

The offspring were weighed and their number recorded after normal parturition as well as after abortions. About 6—8 hours after littering the female rat was weighed and killed. The pituitary glands, ovaries, adrenals, hearts and kidneys were weighed. In case the animals had not littered within 33 days after the onset of

pregnancy they were obdulated so as to ascertain the existence of probable remnants of pregnancy. The body-weights or the weights of the organs were not recorded for these animals.

RESULTS

Body-weights. — The average body-weight in the control group was 225.6 ± 5.4 g at the onset of pregnancy and 230.1 ± 4.9 g after littering. The corresponding weights of the test animals with audiogenic-visual treatment were 233.0 ± 6.9 g and 235.8 ± 5.4 g respectively. The post-parturition weight as compared with the weight observed at the beginning of pregnancy was similar in the test series and in the control group (the increase in weight being about 1 per cent and 2 per cent respectively).

The Weights of the Organs (Table 1 and Fig. 1). — In the test

TABLE 1

THE MEAN WEIGHTS OF THE ORGANS OF RATS (PER 100 G BODY WEIGHT) IN THE CONTROL GROUP AND TEST GROUP AFTER AUDIOPHONIC-VISUAL STIMULI TREATMENT

Group	Adrenal	Pituitary Gland	Ovary	Heart	Kidney
Control ..	16.9 ± 0.8	4.01 ± 0.2	23.3 ± 0.8	352.0 ± 12	359 ± 11.2
Test	18.6 ± 0.7	3.80 ± 0.1	21.5 ± 1.8	359.0 ± 13	394 ± 12.2
P-value	> 0.05	> 0.05	> 0.05	> 0.05	< 0.01

animals the mean weight of the adrenals was a little greater than the respective weights for controls although there was no statistical difference. No significant difference was observable in the weight of the pituitary glands, the ovaries and the heart, but the weight of the kidneys was definitely greater in the test group than in the control series ($P < 0.01$).

Development of Pregnancy. — Of the 15 animals in the test group pregnancy terminated in abortion in 4 cases (about 25 per cent) whereas no abortion took place in the control group. All the abortions occurred during the first half of pregnancy.

The Number and the Weight of the Offspring. — In the control group the number of the offspring varied from 7 to 12 (with a mean of 9) their average weight being 5.3 g. For the 11 rats in the test group whose pregnancy terminated in littering the number of the offspring varied from 6 to 12 (with a mean of 9) the average weight of the offspring being 5.7 g.

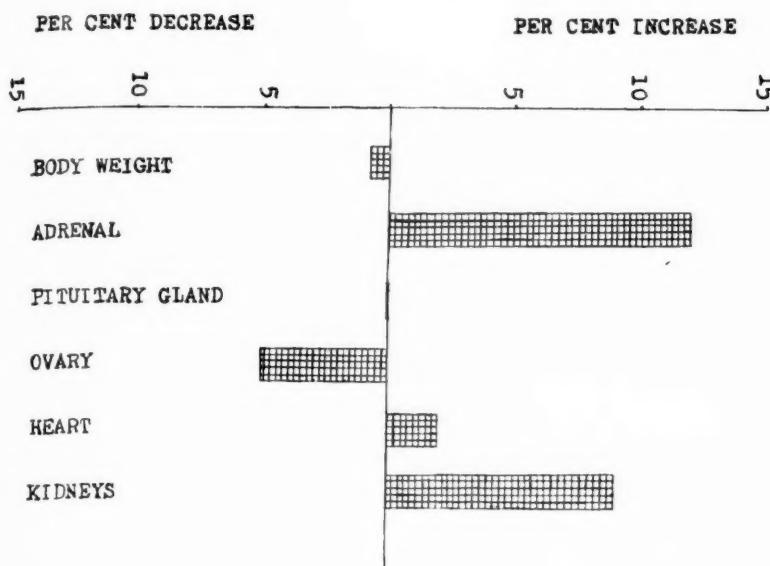


Fig. 1. — The mean percentages changes in organ weights (per 100 g body weight) of the rats of test group as compared with untreated controls. P-values see Table 3.

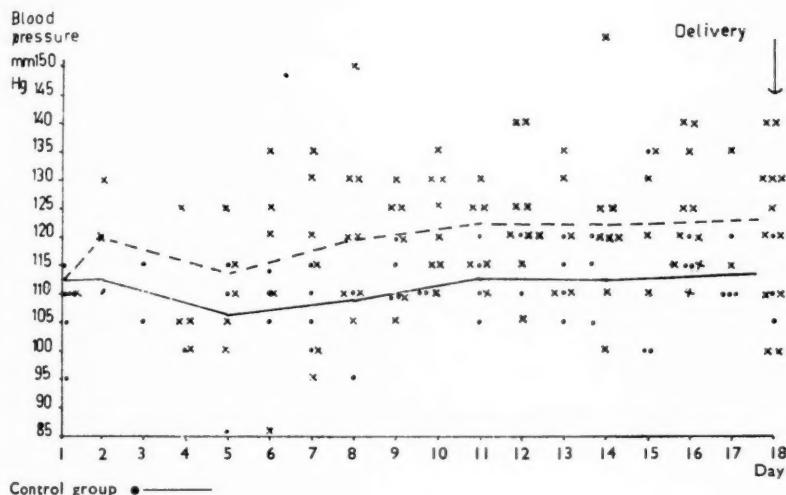


Fig. 2. — The individual and mean blood pressures of the pregnant rats in test and control groups.

Blood Pressure (Fig. 2). — In the control group the blood pressure remained at 112 mm Hg on the average with individual variations ranging from 90 to 135 mm Hg. A pressure of 130 mm Hg or more was observed in only 2 per cent of the controls.

The average blood pressure was on a somewhat higher level in the test series with audiogenic-visual stimuli (123 mm Hg) the individual variations ranging from 85 to 155 mm Hg. A pressure of 130 mm Hg or more was observed in 27 per cent of the test animals. The average blood pressure in the control series was 106 mm Hg on the 5th day of pregnancy and in the test group 112 mm Hg. On the 8th day the average blood pressures were 109 mm Hg and 119 mm Hg, respectively. From the 11th day until the delivery the average blood pressures remained similar; at about 112 mm Hg in the control and 123 mm Hg in the test group.

DISCUSSION

The audiogenic-visual treatment that was used in the present study affected the course of pregnancy; in one fourth of the test animals the pregnancy terminated in abortion. A similar tendency to abortions has also been produced by means of diabetes induced by alloxan injections (2). The audiogenic-visual stimuli also caused a tendency to elevated blood pressure. The mean increase was rather small (from 112 mm Hg to 123 mm Hg on the average), but for some animals the elevation was quite clear (ad 155 mm Hg). It is to be noticed that inducing hypertension in an unsensitized rat is difficult and that an electric shock treatment only produces a slight tendency to elevated blood pressure in e.g. unilaterally nephrectomized rats (4). Still it is true that a long-time audiogenic stimulation is capable of producing hypertension (8). The tendency to hypertension in pregnant rats that have undergone an audiogenic-visual treatment may be produced more likely by the vegetative nervous system and adrenalin than by hydrocortisone (5). An elevated blood pressure in rats can be brought about by means of injections of longacting adrenalin in oil and this effect is still potentiated by sodium chloride and desoxycorticosterone (1). In toxæmia of late pregnancy the noradrenalin infusion causes a more pronounced elevation in blood pressure than in normal pregnancy (6). According to Raab (7) the metabolic catecholamine action seems

to be a fundamental cause of the stress-induced myocardial damage in particular and of the entire «stress syndrome» in general.

In non-pregnant rats an audiogenic-visual treatment produces an increase in the weight of the adrenals, a decrease in the weight of the pituitary gland and the ovaries and a reduction of the gonadal functions (15). A solely audiogenic treatment brings forth similar changes, *i.e.* a more clearly increased adrenocortical and a decreased gonadal activity (9). According to Biró *et al.* (3) audiogenic stimuli produce *e.g.* increase in the weights of the adrenals but they prevent the occurrence of eosinopenia.

Pregnancy was thus unable to prevent the appearance of untoward effects of an audiogenic-visual treatment. It will be of interest to see whether certain drugs are capable of reducing, or preventing, the occurrence of such changes.

SUMMARY

Fifteen pregnant rats were subjected to an audiogenic-visual treatment after the establishing of pregnancy until its termination. In about 25 per cent of the cases pregnancy terminated in abortion. The number and the average weight of the offspring were the same as those obtained for the ten controls. As regards the littered test animals the following results were obtained when weighing the organs: no significant difference was observable in the weight of the adrenals, pituitary glands, ovaries and the heart, but the weight of the kidneys was statistically greater in the test group than in the control series. A slight tendency to elevated blood pressure (a mean of 123 mm Hg) was observable in the test animals as compared with the control group, where the mean was 112 mm Hg.

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